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Characterization of the Suboxic Response in *Brassica napus* L.

by

Lol L.D. Cooper ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

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Department of Plant Science,

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L. Cooper

#9 1601 S.W. 49th Street
Corvallis, Oregon.
97333 USA

July 28, 1997

University of Alberta

Faculty of Graduate Studies And Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Characterization of the Suboxic Response in *Brassica napus* L.** submitted by **Lol L.D. Cooper** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **Plant Physiology**.

Dr. Anne M. Johnson-Flanagan

Dr. Allen G. Good

Dr. Gary S. Stringam

Dr. Maurice M. Moloney

Dr. Arnost Horak

Dr. Greg Taylor

Dr. Malcolm C. Drew
Dept. of Horticulture,
Texas A&M University

Dated: July 28, 1997

This thesis is dedicated to my mother, Penny Maret, who passed away on December 23, 1994, and did not get a chance to see its completion. Her compassion, zest for life and lifelong love of nature, the outdoors and of learning have always been an inspiration.

Abstract

Suboxia is defined as a lower than optimum oxygen concentration, including both hypoxia (slight) and anoxia (severe) stress. The object of this study was to characterize the suboxic response in *Brassica napus* in four different tissues; microspore-derived haploid embryos (MDHEs), germinating seeds, roots of very young seedlings (< four days old) and roots of older seedlings (> four days old). The anaerobic response of the MDHE system was examined with the goal to select for suboxia tolerance *in vitro*. Alcohol dehydrogenase (ADH) levels were compared with changes in the Adenylate Energy Charge (AEC) ratio, the Total Adenylates (TAs) and survival under suboxia stress. Results indicated that regulation of ADH activity was both developmentally and temporally controlled, with a switch occurring during seed germination and early seedling growth. Until two to three days postimbibition in the germinating seeds and young seedlings, and in the MDHE system, there were high levels of ADH enzyme activity which were not inducible under suboxia. Conversely, four day-old seedlings acclimated to root hypoxia, as evidenced by the maintenance of the AEC ratio and the TAs. They responded with slowed growth and a biphasic induction of ADH activity and also developed adventitious roots that showed evidence of schizogenous aerenchyma tissue. High ADH activity in the MDHEs did not result in a maintenance of the TA levels and was not associated with survival under anoxia. Substantial variation in anoxia tolerance was observed in the MDHE population and a selection was made *in vitro*. Unfortunately, this trait was not stable and heritable past the second generation, suggesting that the initial anoxia tolerance observed may have been an epigenetic effect or an example of gametoclonal variation. The implications of this are very important when considering approaches for selecting for flooding tolerance.

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List of Abbreviations

A	adenine
ADH	alcohol dehydrogenase
ADH1	alcohol dehydrogenase-1 protein
***Adh*1**	alcohol dehydrogenase-1 gene
***Adh*2**	alcohol dehydrogenase-2 gene
AdN	adenine nucleotides
ADP	adenosine diphosphate
AEC	Adenylate Energy Charge
AK	adenylate kinase
Ala	alanine
ald	aldolase gene
AMP	adenosine monophosphate
APS	ammonium persulfate
ARE	Anaerobic Regulatory Element
Arg	arginine
AS	Anoxia Survivor line
AS_1	Anoxia Survivor- first generation
AS_2	Anoxia Survivor- second generation
AS_3	Anoxia Survivor- third generation
Asp	aspartate
ATP	adenosine triphosphate
B_5	Gamborg's B_5 medium
bis	N',N'-methylene bisacrylamide
bp	base pair
BRB	Blot Rinse Buffer
BSA	Bovine Serum Albumin
C	cytosine
$C0_2$	carbon dioxide
COPR	Respiratory Critical Oxygen Pressure
Cots	cotyledons
Ctrl	control
DNA	deoxyribonucleic acid
DPA	days post anthesis

EDTA	ethylenediamide tetracacetic acid
EMS	ethyl methanesulphate
F_1	First filial generation
$FADH_2$	flavin-adenine dinucleotide, reduced
$FMNH_2$	flavin mononucluotide, reduced
GABA	γ-amino butyric acid
GLC	Gas-Liquid Chromatography
Glu	glutamate
Gln	glutamine
GPT	glutamate-pyruvate transaminase (alanine amino transferase)
HCl	hydrochloric acid
HPLC	High Performance Liquid Chromatography
HPT	hypoxically pretreated
kb	kilobase
kbp	kilobase pairs
kD	kiloDaltons
Km	Michaelis Menten Constant
Kpa	kilopascals
LDH	lactate dehydrogenase
Lys	lysine
MDHEs	Microspore-derived haploid embryos
mRNA	messenger ribonucleic acid
N_2	nitrogen gas
NAD^+	nicotinamide adenine dinucleotide, oxidized
NADH	nicotinamide adenine dinucleotide, reduced
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NHPT	Non-hypoxically pretreated
NMR	Nuclear Magnetic Resonance
NN	Nitsch and Nitsch Media.
O_2	Oxygen gas

PAGE	Polyacrylamide Gel Electrophoresis
Pdc	pyruvate decarboxylase gene
PDC	pyruvate decarboxylase protein
PEG	polyethylene glycol
PEP	phosphoenol pyruvate
PEPcase	phosphoenol pyruvate carboxylase
PFK	phosphofructokinase
Pi	ortho phosphate
PK	pyruvate kinase
pmol	picomole
pO_2	oxygen partial pressure
Pro	proline
Rads	radicles
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SE	standard error of the mean
Ser	serine
T	thiamine
T_{50}	time to 50% germination
TA	Total Adenylates
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TCA cycle	tricarboxylic acid cycle
Tris	Tris hydroxymethyl, methyl glycine
2,4-D	2,4--dichlorophenoxyacetic acid
Tyr	tyrosine
U	uridine
v/v	volume per volume

Chapter I.

Introduction

Before the rise of the ancestors of the green sulfur bacteria, about 3 billion years ago, microorganisms depended upon fermentative processes to generate energy and reducing power. Later, the early cyanobacteria developed a unique double photocenter which allowed them to use water as a source of hydrogen atoms for carbon dioxide reduction. Consequently, they were able to multiply and oxygen was released into the atmosphere for the first time (Alberts *et al.*, 1989). Modern plants and animals are dependent upon oxygen for survival, but still retain the fermentative pathways which once predominated. The oxidation of one glucose molecule yields 36 ATP from ADP and phosphate, compared to two ATP during substrate level phosphorylation (Davies *et al.*, 1987).

Hypoxic metabolism is characterized by the concurrent activity of both limited respiration (aerobic metabolism) and some degree of fermentation (anaerobic metabolism) (Ricard *et al.*, 1994). Anoxia is restricted to those situations where fermentation can be considered to be the only source of ATP because the production of energy-rich bonds by oxidative phosphorylation has become negligible. When hypoxia reduces respiration to 5% of its initial value, the oxidative production of ATP is, in the absence of the Pasteur Effect, equivalent to that produced by fermentative processes (Pradet and Bomsel, 1978).

Many of our most important agricultural plants, including barley, maize, oats, soybeans and wheat tolerate even the most transient waterlogging poorly (Kennedy *et al.*, 1992). The Crucifereae family of crops has received little attention with regard to flooding tolerance, despite their world-wide importance to agriculture and horticulture. One study from Britain has shown a 15% yield loss as a result of mid-winter waterlogging of winter oilseed rape (*Brassica napus*) (Cannell and Belford, 1980). The anaerobic

response has been well characterized in *Arabidopsis thaliana*, a non-crop plant member of the Crucifereae family (Dolferus *et al.*, 1985; Chang and Meyerowitz, 1986).

Even though flooding is a significant agronomic problem, there are few reports of selection for flooding tolerance. Researchers have improved the flooding tolerance of crops such as *Brassica rapa* (Daugherty and Musgrave, 1994) through selection at the seedling or whole plant stage. Flooding tolerant selections have also been made in wheat (Mian *et al.*, 1993), barley (Hamachi *et al.*, 1990), sorghum (Thseng and Hou, 1993), reed canary grass (Ivanov *et al.*, 1984) and sweet potato (Martin, 1983). The time and space costs of field-scale selection for plants with increased flooding tolerance may be one reason for the lack of interest in this area.

Selection in tissue culture may offer a viable alternative for the development of improved cultivars. These systems offer researchers a variety of advantages; rapid exponential growth, large populations with a limited number of cellular phenotypes or genotypes, freedom from contaminating microorganisms, and savings of time and space over growing whole plants (Dougall, 1980). The microspore-derived haploid embryos (MDHEs) of *B. napus* would make an ideal system to select for anoxia tolerance *in vitro*.

Although there has been little work characterizing MDHEs as a model system for studies of stress-induced responses, they have been used as model systems in other studies. Taylor and co-workers (1990) concluded that MDHEs could potentially be exploited in studies of biochemistry and gene regulation in oilseeds based upon similarities to their zygotic counterparts with regard to storage-lipid and protein composition. Since then, the MDHE system has been used to study freezing tolerance (Orr *et al.*,1990), degreening (Johnson-Flanagan and Thiagarajah, 1990) and gene expression (Wilen *et al.*, 1990; 1991).

Based upon the work of previous researchers (see Literature Review), it was hypothesized that:

1. The ability of a tissue or organism to survive anoxia or hypoxia would depend upon its ability to adapt or acclimate, as well as the severity of the stress.
2. The anaerobic reponse would be mediated by developmental controls during seed germination and early seedling growth.
3. Changes in alcohol dehydrogenase activity would be reflected in changes in the AEC ratio and TA pool under anoxia and hypoxia.
4. The MDHEs could be used to select for anoxia tolerance *in vitro*

To test these hypotheses, the following goals and objectives were set:

Goals and Objectives:

The overall goal was to explore the suboxic response in *Brassica napus*. I wanted to determine the relationships between alcohol dehydrogenase (ADH) activity, Adenylate Energy Charge ratio (AEC), maintenance of the Total Adenylate (TA) pool and survival under anoxia. I also wanted to explore the possibility of using the MDHE system to select for anoxia tolerance *in vitro*. Furthermore, I wanted to examine the possible developmental regulation of the suboxic response in MDHEs, germinating seeds and seedlings.

To this end, the following objectives were set:

1. To characterize the response to long-term hypoxia in seedling roots by examining the changes in growth and development, ADH and the AEC ratio.

-Characterize the effects of hypoxia on seedling growth by following the fresh and dry weight increases in roots and shoots.

-Determine ADH levels in seedling roots under hypoxia through enzyme assays, western blotting and nondenaturing PAGE.

-Examine the relationship between ADH enzyme activity and the amount of ADH protein.

-Examine the development of aerenchyma tissue in adventitious and primary roots in seedlings under long-term hypoxia.

-Characterize the changes that occur in the Adenylate Energy Charge ratio and the levels of TAs during hypoxia treatment.

2. To characterize the anaerobic response in the MDHE system and to determine if it could be utilized to explore the relationship between ADH enzyme activity and survival in anoxia.

-Develop a survival curve to determine how long MDHEs could survive anoxia.

-Determine the pattern of expression of ADH in the MDHE system using enzyme assays, western blotting and nondenaturing PAGE.

-Examine the relationship between ADH enzyme activity and the amount of ADH protein.

-Characterize the changes that occur in the AEC ratios and the levels of TAs during anoxia treatment as related to survival under anoxia.

-Compare the isozymes of ADH active in MDHEs, germinating seeds and seedling roots under anoxia.

3. To determine if the MDHE system could be used to select, *in vitro*, for tolerance to anoxia and whether the selection would be stable and heritable.

-Select for anoxia-tolerant MDHEs *in vitro*, using the Anoxia Culture System.

-Regenerate fertile, diploid plant material and produce seed for characterization.

-Examine the response to anoxia in the regenerated line in comparison to the parental line at the level of the MDHEs.

-Backcross the putative anoxia-tolerant line (AS) with the wild type parental material (T4079) to determine possible pattern of inheritance of the anoxia-tolerant trait and evaluate the anoxia-tolerance of F_1 progeny at the MDHE stage.

-Backcross the F_1 progeny generation to AS and T4079 in reciprocal combinations, and self-pollinate the AS lines, T4079 and the F_1 progeny lines.

-Test the progeny of the backcrosses and self-pollinations at the seedling stage for higher anoxia-tolerance to determine possible pattern of inheritance of the anoxia-tolerant trait.

4. To determine if the suboxic response of the germinating seeds and young seedlings (one to three days old) is developmentally programmed.

-Characterize the pattern of germination in *B. napus* seeds by following the uptake of water.

-Determine the pattern of expression of ADH in the germinating seeds and young seedling roots through enzyme assays, western blotting and nondenaturing PAGE.

-Examine the relationship between ADH enzyme activity and the amount of ADH protein.

-Compare the isozymes of ADH active in germinating seeds and young seedlings under anoxia.

-Determine the inducibility of ADH enzyme activity in the germinating seeds and young seedling under suboxic conditions.

Literature Review

A. Definition of Anaerobiosis, Anoxia and Hypoxia

Hypoxic metabolism is characterized by the concurrent activity of both limited respiration (aerobic metabolism) and some degree of fermentation (anaerobic metabolism) (Ricard *et al.*, 1994). Anoxia or anaerobiosis is restricted to those situations where fermentation can be considered to be the only source of adenosine triphosphate (ATP) because the production of energy-rich bonds by oxidative phosphorylation has become negligible. When hypoxia reduces respiration to 5% of its initial value, the oxidative production of ATP is, in the absence of the Pasteur Effect, equivalent to that produced by fermentative processes (Pradet and Bomsel, 1978).

In practical usage the terms 'anaerobiosis', 'anoxia' and 'hypoxia' are used rather loosely in published works to refer to various states of oxygen deprivation. Variation in treatment conditions as well as the lack of definition of these terms has led to a considerable amount of confusion when the results of various experiments are compared. In many systems that are classified as anaerobic, an inert gas such as nitrogen or argon is bubbled through a liquid growing medium to flush out the available oxygen. Some researchers totally immerse the seedlings or plants while others treat only the roots. As well, some of the experiments are performed in the light with green tissues and some in the dark with green or etiolated tissue. With green tissues, the presence of photosynthetic oxygen may improve the aeration status of the system, thereby creating a less than anaerobic environment. Other terminology such as 'waterlogged', 'flooded', 'under oxygen deprivation' and 'oxygen deficit' are also used in various studies, without adequately defining the extent of hypoxia or anoxia in the experimental system.

For example, Wignarajah and Greenway (1976) used the term 'root anaerobiosis' in their early work on the activities of alcohol dehydrogenase (ADH; E.C. 1.1.1.1) and

pyruvate decarboxylase (PDC; E.C. 1.2.4.1) in roots of *Zea mays* seedlings. In this case, anaerobiosis referred to a situation where O_2-free gas was bubbled through the hydroponic culture solution that the seedling roots were growing in, while the upper portion of the plant was exposed to light and air. Such conditions are probably better classified as hypoxic, but without a means of comparison, it is difficult to assess the severity of the stress. They also use the term 'plant anaerobiosis' when the whole seedling was enclosed in a small glass chamber sealed tightly on the lid of the pot. Nitrogen gas was passed through both the shoot chamber and the nutrient solution, but the plant was in the light for 15 hours, and presumably photosynthesizing.

Sachs and Freeling (1978) 'anaerobically' induced maize seedlings by immersing them for 12 hours in induction buffer (5 mM Tris-HCl, pH 8.0, with 75 *u*g/ml chloramphenicol) then transferring them to an anaerobic chamber where only the primary root was immersed but there was a continuous stream of prepurified, water-saturated N_2 gas. In 1980, Sachs and his co-workers studied the anaerobic proteins of maize by immersing maize seedlings for a specified time period in induction buffer at 30°C in a chamber through which water-saturated argon was continuously bubbled; presumably these treatments occurred in the light since no mention is made otherwise. This method has been adopted by many other researchers (for example; Brzezinski *et al.*, 1986; Dolferus *et al.*, 1985; and Russell *et al.*, 1990).

Rowland and Strommer (1986) describe their experimental system as 'anaerobiosis' when they simply submerged seedlings in buffered water for periods ranging from 1-24 hours. Maize protoplasts, incubated in 5% O_2 and 95% N_2 are described as 'anaerobically induced' in the functional analysis of *in vitro* constructed *Adh*1-CAT genes (Walker *et al.*, 1987). These situations are probably better described as hypoxic.

Some researchers carefully detail the means they chose to stress the plants. For example, Cobb and Kennedy (1987) studied the distribution of ADH in roots and shoots of etiolated rice and *Echinochloa* seedlings under 'anaerobiosis' by placing six day old

seedlings on moist filter paper in chambers continuously flushed with humidified N_2 gas (99.995% purity). All the chambers were maintained at 26°C in the dark. Brown and Beevers (1987) studied the fatty acids of rice coleoptiles in air and 'anoxia' by continuously flushing the seeds with moist N_2 gas (99.998% purity) in sealed, screw cap jars lined with moistened filter papers. The jars were kept in the dark at 25°C. The conditions in these experiments probably are accurately described as anoxic, anaerobic or nearly so.

In some cases where there may be almost no oxygen, researchers have incorrectly used the term 'hypoxia'. Bailey-Serres and Freeling (1990) used the term 'hypoxia' to describe a situation where maize seedlings were completely immersed in an induction buffer in a closed mason jar through which 100% argon was bubbled. In the work of Roberts and his co-workers (1984a; 1984b) the term 'hypoxic' is used to describe a situation where excised root tips of maize seedlings were perfused with an N_2 saturated solution at 4 mL/min "such that the rate of mitochondrial ATP synthesis was less than that due to glycolysis". Both these treatments would probably be better characterized as an anaerobic or anoxic treatment. Peschke and Sachs (1994) use the term 'hypoxic' to describe their system where maize seedlings were immersed in 'drowning buffer' in 500 mL jars that were subsequently sealed and kept in the dark for specified periods of time, with no gas flowing through the buffer. In a situation such as this, it is difficult to tell whether or not these conditions can be defined as either anoxic or hypoxic since there are no data presented as to the concentration of dissolved O_2 in the liquid medium.

There are many examples where the O_2 concentration was measured in the nutrient solution using various O_2 analyzing equipment. For example, Thomson and co-workers (1989) grew three day old wheat seedlings in a nutrient solution through which was passed mixtures of air and N_2 gases that resulted in O_2 concentrations ranging from 0.01 to 0.26 mol/m^3 (0.32-8.32 mg O_2/L) as measured by a Beckman Fieldlab O_2 analyzer. Hanson and Jacobsen (1984) studied the activity of ADH and lactate dehydrogenase (LDH; E.C. 1.1.1.27) in barley aleurone layers by incubating excised layers in incubation

medium in flasks in a desiccator that was purged with N_2 gas, then sealed. Mixtures of O_2 and N_2 were achieved by injecting O_2 and simultaneously withdrawing N_2. In this case the concentrations of O_2 and N_2 were analyzed by gas-liquid chromatography of the headspace gas, and the concentration of O_2 was reported to range from 0-20%.

There are many examples in the literature of the terms 'waterlogged', 'flooded', or 'inundated'. For example, Cannell and Bedford (1980) studied the effects of 'waterlogging' in lysimeters on the growth and yield of oilseed rape (*B. napus*) by watering the lysimeters to the soil surface for various periods of time. Another 'waterlogging' experiment looked at the growth response of mungbeans grown in pots inside acrylic boxes when the acrylic boxes were filled with water up to the surface of the growing medium in the pots (Musgrave and Vanhoy, 1989). In these experiments there was no measure of the severity of the oxygen stress or means to compare it with other results.

In cell culture experiments 'anaerobic' conditions have been created in various ways. Vanlerberghe and Turpin (1990) treated the green algae *Selenastrum minitum* with an O_2 scavenging system and by blowing N_2 gas into the headspace of the cuvettes. *Medicago sativa* cell suspensions were treated with 'anaerobic' conditions by completely filling the culture vial with the cell suspension and sealing the screw cap. Subsequently, the vials were covered with aluminum foil and were shaken at 150 rpm in the dark (Lai *et al.*, 1992). Widholm and Kishinami (1988) used the term 'anaerobic' to describe the conditions in *Nicotiana plumbaginifolia* cell cultures when they were not shaken. The latter two examples are probably more like a hypoxic stress than one of anoxia.

Johnson and co-workers (1994) used the terminology of Pradet and Bomsel (1978), by which tissues or cells are defined as hypoxic when the O_2 partial pressure limits the production of ATP by the mitochondria. Anoxia is defined as a situation where the O_2 partial pressure is so low that ATP production by oxidative phosphorylation is negligible compared to that generated by glycolysis and fermentation. Their system involved placing

etiolated maize seedlings into expanded polystyrene floats in sealed, screw-top jars with entrance and exit ports in the lids. The jars were kept in the dark and the nutrient solution in the jars was continuously sparged at 200 mL/min with either air, prepurified N_2 gas (99.997% purity) or with various O_2/ N_2 mixtures. The roots were in contact with the solution while the coleoptile and the first emerging leaf were in the gas in the headspace. They state "the concentration of the dissolved O_2 in the solution is directly proportional to the O_2 partial pressure in the gas mixture used to sparge the solution and that the concentration of O_2 in water in equilibrium with moist air at 25°C (21.4% v/v O_2) is 258 *u*M and the presence of dilute calcium sulphate in the solution has negligible effects upon the O_2 solubility". They use the following definitions: hypoxia- 4% O_2 (v/v), aerobic conditions- 40% O_2 and anaerobic conditions- 99.997% N_2. The same experimental design was used in previous work (Hole *et al.*, 1992; Andrews *et al.*, 1993; Andrews *et al.*, 1994a; Andrews *et al.*, 1994b; He *et al.*, 1994; and Johnson *et al.*, 1994).

Definition	% O_2	*u*M	mol/m^3	mg O_2/L	pO_2 (kPa)
Aerobic (Johnson *et al.*, 1994)	40	481	0.481	15.5	40
Normoxia (Johnson *et al.*, 1994)	21.4	258	0.258	8.3	21.4
Hypoxia (Pradet and Bomsel, 1978)	4	46.6	0.046	1.5	4
Anaerobic (Johnson *et al.*, 1994)	0.003	0.037	3.7×10^{-5}	0.0012	.003
(Thomson *et al.*, 1989)	0.81 - 21	10 - 260	0.01 - 0.26	0.32 - 8.32	0.81 - 21

Table 1-1. A comparison of the terminology used to describe oxygen concentration.

The above table compares the terminology used to describe various treatment conditions in the literature.

In addition to the problems stated above, there is published evidence that a gradient of O_2 deficiency may exist across tissues exposed to low O_2 partial pressures. This may be another confounding factor in estimating the degree of oxygen stress imposed upon plant tissues. Diffusion of O_2 through plant tissues is limiting, and a decreased rate of respiration at progressively lower pO_2 is a result of an increasing core of anaerobiosis in the system (Berry and Norris, 1949). For instance, in maize seedling roots exposed to low external oxygen concentrations, it was found that the stele receives inadequate O_2 for oxidative phosphorylation, while the cortex continues to respire, even when the external solution is at zero O_2. These results suggested that for roots in an O_2-free solution, aerenchyma provided adequate O_2 for the respiration in the cortex, but not in the stele. When the supply of O_2 to the aerenchyma was blocked, this effect was eliminated (Thomson and Greenway, 1991).

Armstrong and co-workers (1991) developed a multicylindrical model of root aeration by diffusion. Data derived from the model demonstrated the wide diversity in respiratory critical oxygen pressure (COPR) which possibly varies not only between species or between roots, but also, within an individual root, with time. The COPR is defined as the lowest oxygen partial pressure that fails to retard oxygen consumption. It was shown that the COPR could vary from less than 2 kPa to values greater than 100 kPa. Using this model, they predicted that O_2 gradients across the root are gradual in the cortex because of this tissue's low O_2 demand and high porosity and are steep in the stele because of its higher O_2 demand and lower porosity. They concluded that in aerenchymatous graminacean roots, which are submerged and growing, anoxia is most likely to occur first in the central parts of the stele and/or meristem. The stelar anoxia probably occurs first in the apex and spreads laterally and basally with further root extension. Ethanol production, raised ADH activity and the reduced energy charge in roots under anoxia might be a function of sub-apical stelar anoxia only. The root apices are especially

susceptible to flooding because the meristem tissues are compact and lack intracellular spaces (Vartepetian, 1978). As well, the root tips have a much greater respiratory demand because they are the site of cell division and expansion.

B. Roles of Oxygen in Plant Survival

1. Energy production

Plant cells, like animal cells, require oxygen for a number of metabolic processes. Oxidative phosphorylation is the most important function of oxygen in both plant and animal cells. During the breakdown of carbohydrates, fats and amino acids, electrons flow from the organic substances to oxygen, yielding energy for the generation of ATP from adenosine diphosphate (ADP) and phosphate.

The generation of ATP occurs in mitochondria, on the inner mitochondrial membrane, where the most important part of the respiratory chain, the enzyme complex ATP synthase, resides. According to the Chemiosmotic Hypothesis proposed by Mitchell (1966), a pH gradient is created across the inner mitochondrial membrane by the H^+ ions that are pumped from the mitochondrial matrix to the intermembrane space by the electron transport chain. The potential energy inherent in this gradient was postulated to be the driving force behind the energy-requiring synthesis of ATP from ADP (Alberts *et al.*, 1989).

In plant cells, yeast, and some other microorganisms under anaerobiosis, glucose is primarily fermented to ethanol and CO_2 (van Waarde, 1991). Anaerobic glycolysis also occurs in animal cells during short bursts of intense activity, during which oxygen cannot be carried to the muscle fast enough to sustain the oxidation of pyruvate to generate ATP. Stored glycogen in muscle cells is broken down to lactate, which is converted by the liver to pyruvate, and eventually to glucose during the rest or recovery period (Hochachka, 1986). During substrate level phosphorylation (alcoholic fermentation), glucose is broken down to two pyruvate molecules yielding only two ATP and the pyruvate is further

broken down to either acetaldehyde and then to ethanol by the enzymes PDC and ADH, or to lactate by lactate dehydrogenase (LDH). The reducing power for the dehydrogenase reaction comes from glyceraldehyde 3-phosphate dehydrogenation that produces NADH.

$$CH_3 - CO - COOH \xleftrightarrow{PDC} CH_3 - CHO \xleftrightarrow{ADH} CH_3 - CH_2OH$$

Thus for one glucose there is a yield of only four ATP molecules, and the NADH originally formed during the conversion of glyceraldehyde 3-phosphate to 3-phosphoglycerol phosphate does not enter the electron transport chain. During oxidative phosphorylation, the total possible ATP yield is 38 ATP per glucose molecule entering glycolysis. When the two priming reactions are taken into account, the oxidative phosphorylation of one glucose molecule gives a net yield of 36 ATP from ADP and phosphate, compared to a yield of two ATP during substrate level phosphorylation (Davies *et al.*, 1987), only ~5% of the yield. When the fermentative energy yield is compared to the energy yield from oxidative phosphorylation, it is obvious why an oxygen-free environment would require special metabolic and morphological adaptations to enable plants to survive.

2. Oxygen in metabolic pathways and organic compounds

Although the activity of cytochrome oxidase accounts for over 90% of the O_2 consumed by most cells, some oxygen is incorporated into organic substrate molecules, for instance, forming new carboxyl or hydroxyl groups. On the whole, oxygen is an important component of many organic substances including amino acids, fatty acids, nucleotides, and carbohydrates. It has been estimated that oxygen makes up about 78% of the fresh weight of plant cells on the basis of atomic composition (Raven *et al.*, 1986). Specialized enzymes in some cells carry out oxidative reactions in which either one or both of the oxygen atoms of water are incorporated into organic substrates. Dioxygenases incorporate both atoms of oxygen into the new molecule. One example of this kind of enzyme is pyrocatechase, which catalyzes the opening of the catechol ring by reaction with O_2, forming two carboxyl groups. Monooxygenases, which are more common and more

complex in their action, catalyze reactions where one of the two atoms of oxygen is incorporated into the organic substrate and the other atom is reduced to water. Two different types of monooxygenases exist, differentiated on the basis of whether they obtain the reducing power for these reactions from NADH or NADPH or from the reduced flavin nucleotides ($FMNH_2$ and $FADH_2$). For example, phenylalanine 4-monooxygenase (E.C. 1.14.16.1) catalyzes the hydroxylation of phenylalanine to form tyrosine, with reducing power provided by NADPH (Lehninger, 1982).

C. The Occurrence of Anoxia and Hypoxia

1. Anoxia or hypoxia during seed germination and seedling growth

During the process of seed germination, several steps occur that culminate in the protrusion of the radical through the seed coat. First, the seed takes up water from its environment in the process of imbibition. This switches the seed from a very low level of metabolic activity to a state of active growth. During this process energy is required for the synthesis of enzymes to degrade stored reserves such as starch or lipids, and for the synthesis of proteins, nucleic acids and other cellular components necessary for early growth. Respiration increases dramatically during the first hours of imbibition as these processes take place (Bewley and Black, 1994). Whether or not a seed can successfully germinate depends upon whether there is enough energy to support this high respiratory demand.

Anoxic or hypoxic conditions are thought to occur inside the seed coat of some seeds that have gas-impenetrable seed coats, and/or if the seeds are in waterlogged or flooded soils (Bewley and Black, 1994). It is generally believed that, during the first hours of imbibition, most seeds are under a 'natural anaerobiosis', since even when the conditions are well-aerated, there may be some accumulation of ethanol and lactate (Raymond *et al.*, 1985). This period is also characterized by high activities of glycolytic enzymes (van Waarde, 1991). Although the affinity of cytochrome oxidase for O_2 is extremely high, (Km 0.1 *u*M, ~0.008 kPa), respiration of many plant tissues and organs

may become limited at a pO_2 closer to that of air (~21 kPa), which is several orders of magnitude higher. For example, the critical pO_2 for the respiration of lettuce seeds during the second phase of germination is about 15 kPa. This has been explained by the presence of barriers to the diffusion of O_2 from the surface of the seed, to the inner membranes of the mitochondria (Ali-Ani *et al.*, 1985).

Most higher plant seeds fail to germinate under low oxygen availability, and only a few, such as rice (Cobb and Kennedy, 1987), and some weeds of the *Echinochloa* genus (Rumpho and Kennedy, 1983; Cobb and Kennedy, 1987) can germinate in a completely oxygen-free environment (Perata *et al.*, 1992). Ethanol is the main metabolic product in these species under anoxic conditions (Bertani *et al.*, 1980, Cobb and Kennedy, 1987). During anaerobic germination, shoots are formed, but root development is suppressed. As soon as the shoot reaches an aerobic zone, O_2 is transported downward and root growth starts (Cobb and Kennedy, 1987).

Fermentation is very active during the initial stages of germination of some seeds. For instance, Leblova (1978a) found that many mono- and dicotyledonous plants with proteins, starch and fats as reserve substances form ethanol during the first three days of germination. The concentration of ethanol was measured during imbibition of bean (*Phaseolus vulgaris*), broad bean (*Vicia faba*), lentil (*Lens esculenta*), pea (*Pisum sativum*), lupine (*Lupinus albus*), soybean (*Glycine max*), barley (*Hordeum distichon*), maize (*Z. mays*), wheat (*Triticum aestivum*), oat (*Avena sativa*), rye (*Secale cereal*), cucumber (*Cucumis sativus*), melon (*Cucumiis melo*), flax (*Linum usitatissimum*), sunflower (*Helianthus annus*), and rape (*B. napus*, var. arvensis). The maximum concentration of ethanol was generally found between 30 and 60 hours after imbibition, after which it decreased. The activity of ADH increased during the first hours of germination and attained a maximum, characteristic for the type of seed, usually between 24 to 48 hours, after which the activity declined (Leblova, 1978b).

Even if seeds are able to germinate, the seedlings are susceptible to both root anoxia and hypoxia. Many plants endure seasonal flooding, resulting in oxygen deficiency. In extreme cases, whole seedlings may be submerged. If a seedling is growing in flooded soil, the factor limiting respiration becomes the diffusion of oxygen into the soil. The diffusion of oxygen in water is 100 times slower in water than in air (Thomson and Greenway, 1991).

The roots of some plants are able to obtain oxygen from the aerial portions by diffusion through intracellular spaces. Oxygen transport has been demonstrated in the roots of cultivated plants such as corn (*Z. mays*), pea (*P. sativum*), barley (*Hordeum vulgare*) and cotton (*Gossypium hirsutum*) (Pradet and Bomsel, 1978). There is considerable evidence that internal, longitudinally connected, intercellular spaces caused by cell separation (schizogenous aerenchyma) or by breakdown of cells (lysigenous aerenchyma) in the cortex aids root growth and survival in oxygen deficient surroundings. These lacunae facilitate gas diffusion between the root and the aerial portion of the plant, while also decreasing the total O_2 demand (Jackson, 1985). This phenomenon is present in many hydrophytes (Justin and Armstrong, 1987) and in adventitious roots of maize (Drew *et al.*, 1979; 1980; 1981; 1985; Konings, 1982; Konings and de Wolf, 1984) and rice (Jackson *et al.*, 1985). Rhizomes of marsh plants, such as bulrush (*Schoenoplectus lacustris*), reed mace (*Typha angustifolia*) and reed sweet-grass (*Glyceria maxima*) are also adapted to anaerobic conditions (Crawford, 1978; Monk and Braendle, 1982)

Perennial field crops such as alfalfa (*M. sativa*) or winter annuals such as winter wheat (*T. aestivum*) may be subjected to anoxia stress during the winter by the formation of ice crusts on the soil or on the plants themselves. For example, rosette-type overwintering plants in northern temperate areas may be subjected to low-temperature flooding or snowmelt, which can result in ice encasement of the plants upon refreezing. During mid-winter thaws or rain, water percolates through snow layers to build up at the soil surface and freeze over the plants with the return of normal subfreezing winter temperatures (Andrews and Pomeroy, 1989). This condition normally results in severe

damage as a result of the restriction of gaseous exchange and the accumulation of anaerobic metabolites that interfere with cellular metabolism and function, such as ethanol, CO_2 and low levels of lactic acid, which can be toxic to the plant and may reduce subsequent cold hardiness (Andrews and Pomeroy, 1989).

2. Aquatic plants

Studying plants that are well adapted to an aquatic environment can help us understand how terrestrial plants may adapt or respond to flooding. Wetland ecosystems support a wide diversity of plant species around the world. Anaerobic conditions prevail to a greater or lesser extent in all wetlands and are important in determining the environment within which other processes and organisms operate. Wetlands are defined in various ways but generally are characterized by at least periodic saturation with water, the development of hydric soil types and an association with highly specialized hydrophytic vegetation (Maltby, 1991). These definitions of wetlands include marshes, swamps and peat-forming bogs and fens.

Aquatic plants demonstrate the presence of fermentative pathways during treatment with anaerobic conditions. Many studies have shown the induction of the enzymes ADH and LDH in different flood-tolerant plants. For example, in root apices of *Ranunculus sceleratus*, *G. maxima* and *Senecio aquaticus*, the maximum catalytic activity of ADH increased from 1.5 to 5 fold following reduced aeration of the roots (not strict anoxia) (Smith and ap Rees, 1979a). There was an increase in LDH activity in *G. maxima* and *S. aquaticus*, but no increase in phosphoenolpyruvate carboxylase (PEPcase; E.C. 4.1.1.31) or phosphofructokinase (PFK; E.C. 2.7.1.11) in any of the three. Metabolism of [U-^{14}C] sucrose under anaerobic conditions by excised roots, grown without aeration, led to appreciable labeling of ethanol and alanine, slight, but significant labeling of lactate, and no detectable labeling of malate.

In another study, excised, anoxic roots of the flood-tolerant plants *R. sceleratus*, *S. aquaticus* and *G. maxima* were compared with those of the flood-intolerant plant *P.*

sativum (ap Rees *et al.*, 1987). They found that in all four of the plants the pathways of fermentation were similar; they all depended primarily on ethanolic fermentation, and malate was not a significant product in any of them. There was no significant difference in the amount of induction of ADH between *G. maxima* and *P. sativum*. No significant change was detected in pea roots and there was only a slight rise in ADH activity in *G. maxima*.

In rhizomes of bulrush (*S. lacustris*), it was shown that O_2 consumption and activity of the enzymes cytochrome oxidase, ADH, and LDH are able to adapt to changing levels of O_2. When the rhizomes were gassed with pure N_2 for three days, there was a 2.5 fold increase in the activity of the fermentative enzymes, ADH and LDH and a corresponding decrease in activity of cytochrome oxidase (E.C. 1.9.3.1) (Monk and Braendle, 1982).

Mendelssohn and McKee (1987) showed that root hypoxia ($[O_2]$ = 0.92 +/- 0.05 mg/L) of *Spartina alterniflora* caused significant increases (from 4.7 to 6 fold) in the activity of root ADH. This indicated that the well-developed aerenchyma system of this plant was not capable of providing sufficient aeration to maintain complete aerobic respiration. There was very little accumulation of ethanol, presumably as a result of diffusion into the medium, and there was a small increase in the levels of malate.

Acorus calamus is a monocotyledonous wetland plant that can withstand extremely long periods of anoxia. Bucher and Kuhlemeier (1993) studied the expression of the genes coding for pyruvate decarboxylase (*Pdc*), alcohol dehydrogenase (*Adh*) and fructose-1,6-bisphosphate aldolase (*ald*; E.C. 4.1.2.13) during periods of anoxia ranging from two hours to two months. Upon anoxic induction, *Pdc* mRNA levels peaked at six hours, followed by *Adh* and *ald*, which reach their maximum levels at 12 and 72 hours, respectively. Subsequently, the mRNA levels of all three genes declined within days to low levels. Protein levels of ADH increased steadily for at least a week, then remained

constant. Native gel electrophoresis showed the presence of two sets of ADH isozymes, one present constitutively and one set enhanced under anoxia.

In another study, rhizomes of *A. calamus*, *G. maxima*, *Phragmites australis*, *S. lacustris* and *T. latifolia* were incubated for 72 hours in a closed system initially containing 21% O_2, 1% O_2 or N_2 gas (Studer and Braendle, 1987). Gas samples from the headspace were analyzed daily by gas chromatograph to determine the levels of ethanol, acetaldehyde and ethylene. The rhizomes of all the species produced ethanol and acetaldehyde under anoxia, to a lesser extent under hypoxia, and *S. lacustris* even produced these under normoxia (21% O_2).

The effects of oxygen concentration on seed germination was examined in several species of the *Echinochloa* complex (barnyard grass), a large and diverse group of grasses that are the worst weeds in rice fields world-wide, whether in upland (non-flooded) or lowland (flooded) culture (Kennedy *et al.*, 1987). Within the *Echinochloa* complex there is a wide range of flooding tolerance. For example, *E. crus-galli* var. *oryzicola* (hereafter oryzicola) only occurs as a weed in the flooded rice fields while *E. crus-galli* var. *crus-galli* (hereafter crus-galli) only grows on the dry levees surrounding the paddies. A third species, *E. muricata*, which has intermediate tolerance, is found along streambanks (Barrett and Wilson, 1981). Kennedy and co-workers (1987) studied the germination physiology of these two species, as well as rice (*Oryza sativa*) and *E. muricata*, under different temperature and oxygen concentrations. Under anaerobic conditions (0% O_2), oryzicola reached T_{50} (number of days required for 50% of the seeds to germinate (coleoptile emergence)) after about 10 days at 19°C, while in rice the T_{50} was 10.5 days and in crus-galli it was 11.5 days.

Kennedy and co-workers (1987) studied the germination physiology of oryzicola and crus-galli under different oxygen concentrations. They found that there was no correlation between flood tolerance and ethanol production, ADH activity, or number of ADH isozymes, but they observed that in the seeds germinated anaerobically, an additional

ADH isozyme was induced, which was correlated with increased ADH activity. Furthermore, the flood-tolerant species were able to synthesize adenylates under anoxia, while the flood-intolerant species were not.

In an earlier study, Kennedy and co-workers (1980) observed that crus-galli and oryzicola germinated as well as rice under anaerobiosis. Ultrastructural studies of mitochondria from anaerobically-grown indicated that they were virtually indistinguishable in size and shape from those in aerobically grown oryzicola. Although they did not observe a Pasteur Effect in these species, active metabolism was indicated under anaerobiosis in scanning electron micrographs of oryzicola endosperm starch granules, by the layered, partially digested appearance of the starch, ultrastructural evidence of starch breakdown and utilization. The active metabolism of anaerobically-grown seedlings of oryzicola may in part explain its ability to germinate and emerge from flooded rice fields.

Rumpho and co-workers (1984) measured the Adenylate Energy Charge ratio in oryzicola seeds germinated in an aerobic or anaerobic environment. Within 24 hours after imbibition, total nucleotides increased and energy charge values increased from 0.6 to 0.8 in both air and N_2. If the seedling were imbibed in air and subsequently transferred to N_2, the energy charge initially decreased, but subsequently (within 24 hours) increased to at least 0.8. The ability of this plant to maintain a high level of energy under anoxia is one of the adaptations that allows oryzicola to germinate and grow under anaerobic conditions. In its natural environment, this would sustain coleoptile growth and penetration of the shoot into a more aerobic zone.

3. Hypoxia in cell cultures

Plant cell cultures are utilized for many studies of plant metabolism, development and physiology. There are a wide variety of systems, from the growth in culture of primary explants, callus cultures, and cell suspension cultures to the culture of somatic or gametophytic embryos. These systems offer researchers a variety of advantages; rapid

exponential growth, large populations with a limited number of cellular phenotypes or genotypes, freedom from contaminating microorganisms, and savings of time and space over growing whole plants (Dougall, 1980). Cell suspension cultures and microspore-derived haploid embryos (MDHEs) grown in liquid nutrient media must be agitated to provide oxygen to the cells for aerobic respiration. Since these cells are growing in an environment of decreased oxygen diffusion, some degree of hypoxia or anoxia would be expected.

In their study of anaerobic stress in alfalfa (*M. sativa*) cell suspension cultures, Lai and co-workers (1992) observed that there was a significant quantity of ADH activity in the suspension culture cells prior to anoxia treatment. As well, Widholm and Kishinami (1988) observed that *Nicotiana plumbaginifolia* cultured cells had significant ADH activity that increased over threefold when the culture was not shaken. Callis and co-workers (1987) observed that cultured maize cells were appropriate for the study of *Adh*1 expression because the *Adh*1 gene is normally expressed in these cells. The presence of endogenous ADH activity in these cells may indicate a degree of hypoxia inherent in the system. However, in all cases, hypoxia or anoxia further increased the amount of ADH activity. This fact is important to consider when utilizing such systems for metabolic studies.

During the process of MDHE culture, microspores are isolated from immature buds and usually cultured in a liquid medium with high levels of sucrose (Chuong and Beversdorf, 1985). Very little work has been done on the metabolic state of such embryos, but in comparison to the observations made in cell suspensions, it would not be unexpected to find that the MDHEs in culture are also experiencing some degree of hypoxia or anoxia. In addition, the tissue mass of a haploid embryo is much greater than that of an isolated cell in suspension, so there would be considerably more diffusive resistance to oxygen penetrating to the inner layers of cells.

D. Theories of Tolerance to Anoxia or Hypoxia

1. Crawford's Metabolic Theory of flooding tolerance

An early attempt to explain flooding tolerance was proposed by McManmon and Crawford (1971) in their 'Metabolic Theory' of flooding tolerance. They felt that a plant's ability to withstand periods of anoxia stress depended upon decreased ethanol production as a result of low ADH enzyme activity. This resulted in a decrease in the presumably toxic effects of ethanol accumulation and the production of alternate end products such as malate, lactate or other organic acids. This theory has generally been disproven by subsequent work (for example; Smith and ap Rees, 1979a; Rumpho and Kennedy, 1983; ap Rees *et al.*, 1987; Kennedy *et al.*, 1992).

2. The Davies- Roberts pH Stat hypothesis

The Davies- Roberts pH Stat hypothesis involves the relative rate of synthesis of lactate versus ethanol, under the control of the cytoplasmic pH. Under anaerobic conditions, pyruvate is initially converted to lactate, but as the cytoplasmic pH decreases, LDH activity is inhibited, and the activities of pyruvate decarboxylase PDC and ADH are stimulated and ethanol is produced. This theory was originally proposed by Davies (1980) and has been supported by Roberts and co-workers (1982; 1984a; 1984b; 1985; 1989). Using noninvasive ^{31}P and ^{13}C techniques, they showed that during the first 20 minutes of anoxia, the cytoplasmic pH in maize root-tip cells decreased from 7.3 to 6.8 before leveling off. In truly flood-tolerant plants, such as rice or *E. phyllopogon*, the pH declined only transiently, then recovered (Kennedy *et al.*, 1987). Menegus and co-workers (1989; 1991) observed similar phenomena in their survey of several resistant and sensitive plants. They found that anoxia caused an overall acidification of the cytoplasm in sensitive plants such as wheat and barley, but alkalinization of the cytoplasm occurred in resistant plants such as rice and *Echinochloa*.

The metabolic control of anaerobic glycolysis was examined by Rivoal and Hanson (1994) in their study of the overexpression of LDH in transgenic tomato roots. Constitutive expression of the transgene had no appreciable effect on fermentative metabolism in either hypoxically induced or uninduced roots. In both sysems, lactate accumulation slowed markedly after the first hour of anoxia. Their results ndicated that the capacity to secrete lactate to the medium may be one factor in the control of lactate fermentation, which is not consistent with the Davies- Roberts pH Stat hypothesis

F. The Pasteur Effect and the Regulation of Glycolysis and Fermentation

1. The Pasteur Effect

There is a direct regulatory link between the glycolytic system and the aerobic respiration system. Glycolysis precedes the aerobic stage and prepares organic substrates for the next phase of breakdown and oxidation. When a cell is deprived of molecular oxygen, the aerobic respiration system is switched off and the breakdown of respiratory substrates in glycolysis may be accelerated. The Pasteur Effect is the acceleration of glycolysis and fermentation to meet cellular needs for energy and reducing power when the supply of oxygen is low (Warburg, 1926). Also known as 'the conservation of carbon by oxygen', it was first elucidated by Louis Pasteur in 1861. He observed that yeast cells, when subjected to decreasing concentrations of O_2 in the atmosphere, underwent some kind of change, where they produced more CO_2, consumed more carbohydrates and grew more slowly. He also observed that under these conditions they produced ethanol as a metabolic waste product. When the same cell was returned to an oxygen environment, the rate of organic matter consumption was greatly reduced. Intuitively, this makes sense, since we know that organic substrates are utilized much more efficiently in the aerobic pathways (Lehninger, 1982). Ethanol, acetaldehyde and other glycolytic intermediates are the main products of fermentation in plant tissues. As well, there is a transient increase in lactate and increases in alanine (in the presence of an adequate nitrogen source) (Kohl *et*

al., 1978; Bennett and Freeling, 1987). The Pasteur Effect has been observed in many plants, including flooding-tolerant, aquatic species (Jenkins and ap Rees, 1983; Smith and ap Rees, 1979a) and susceptible ones (Smith and ap Rees, 1979b).

2. The regulation of glycolysis and ethanolic fermentation

The classical definition of glycolysis is the conversion of one mole of glucose into two moles of pyruvate (Miernyk, 1990). The regulation of glycolysis and its interaction with the regulation of the aerobic respiratory pathways involves the enzyme PFK that catalyzes the conversion of fructose-6-phosphate into fructose-bisphosphate. The activity of this enzyme is allosterically controlled by a number of metabolites, among which are ATP, AMP, ADP, citrate and inorganic phosphate. Phosophofructokinase from higher plants is inhibited by all three adenine nucleotides (AdNs), especially ATP, which is a powerful allosteric inhibitor of PFK. When mitochondrial respiration is reduced under anoxia, the concentration of ATP drops sharply, so glycolytic processes are increased. In addition, inorganic phosphate is an allosteric activator of PFK (Vartepetian, 1978).

Citrate, phosphoenol pyruvate and phosphate are considered the main effectors of plant PFK (Turner and Turner, 1975). Citrate, synthesized in the tricarboxylic acid cycle under aerobic conditions, is an allosteric inhibitor of PFK. When the supply of O_2 is low or there is none, the tricarboxylic acid cycle is naturally shut off or greatly reduced. As a result, PFK is no longer inhibited by citrate. This allows phosphorylation of fructose-6-phosphate, providing a larger amount of substrate for glycolysis and fermentation (Lehninger, 1982). An increase in the amount of inorganic phosphate in the cell from the utilization of ATP will also contribute to the activation of PFK and the acceleration of glycolysis (Vartepetian, 1978).

Pyruvate kinase (PK; E.C. 2.7.1.40) from higher plants, like that from most organisms, is inhibited by ATP (Turner and Turner, 1975). Pyruvate kinase from cotton seeds is activated by AMP (Duggleby and Dennis, 1973), but AMP has no effect upon the PKs of pea seeds or carrot tissues (Turner and Turner, 1975), soybean nodules (Peterson

and Evans, 1978) and germinating castor bean endosperm (Ireland *et al.*, 1980). However, AMP does inhibit the cytosolic PK isoenzyme of the developing castor bean endosperm (Ireland *et al.*, 1980).

Regulation of ethanolic fermentation occurs at a number of levels. The reaction catalyzed by PDC is considered the rate-limiting step in the ethanol pathway (van Waarde, 1991). Davies and co-workers (Davies *et al.*, 1974; Davies, 1980) examined the factors controlling lactate and ethanol production in cell-free extracts prepared from pea seeds and parsnip (*Pastinaca sativa*) roots. They showed that PDC has an acid pH optimum, whereas LDH performs optimally in the alkaline range. The adenylate nucleotides (ATP, ADP and AMP) do not affect PDC, in contrast to LDH, which is inhibited by ATP, especially at low pH values. At the onset of anoxic exposure, the NADH/NAD^+ ratio increases and lactate accumulates. The resulting acidosis activates PDC and initiates competition for pyruvate. When the intracellular pH reaches 6.9, inhibition of LDH by ATP becomes very important, the production of lactate virtually stops and ethanol becomes the predominant anaerobic end product (van Waarde, 1991).

The work of Roberts and his co-workers (1984a) confirmed the Davies hypothesis that H^+ ions were the signal triggering ethanol production. They observed that anoxia caused a rapid (less than two minutes) increase in NADH, followed by a transient (about 30 minutes) accumulation of lactate. After 35 minutes, the pH and lactate concentrations stabilized as a result of activation of the ethanol branch of the fermentation pathway. When the pH of maize root tips was manipulated prior to anoxic treatment, the lag time preceding the production of ethanol was abolished.

F. Alternative Pathways of Metabolism under Anoxia

1. Changes in organic acids

In addition to the compounds produced during alcoholic fermentation (ethanol and lactate), other metabolic intermediates have been known to accumulate under anaerobic

stress. Some tolerant plant species delay or avoid accumulation of ethanol by diverting glycolytic intermediates to alternate end products such as malate, succinate and alanine (Kennedy *et al.*, 1992). Accumulation of malate or succinate permits reoxidation of pyridine nucleotides (Crawford, 1978). Mendelssohn and co-workers (1981) observed significantly higher malate concentrations in roots of flooded plants of *S. alterniflora*, than in those of nonflooded plants. The higher malate levels in the flooded plants were correlated with higher ADH activities and ethanolic fermentation. However, various researchers have observed that malate concentrations actually slowly decrease under anoxia (Saglio *et al.*, 1980, Menegus *et al.*, 1989, Rivoal *et al.*, 1989; Vanlerberghe *et al.*, 1990).

Smith and ap Rees (1979a) examined the pathways of carbohydrate fermentation in unaerated roots of three aquatic plant species; *R. sceleratus*, *S. aquaticus* and *G. maxima*. Metabolism of [U-^{14}C] led to appreciable labeling of ethanol and alanine, a slight but significant labeling of lactate, but minimal labeling of malate and related organic acids. In a later study, the pathways of fermentation were studied in excised, anoxic roots of the flood-intolerant plant, pea, *P. sativum* (ap Rees *et al.*, 1987). They found that pea roots under anoxia depended primarily on ethanolic fermentation, and malate was not a significant product.

2. Changes in alanine levels and free amino acids under anoxia

The pathway of alanine (Ala) synthesis in plant tissues involves the transfer of the amino group from glutamate (Glu) to pyruvate by alanine aminotransferase (E.C. 2.6.1.2) (also called glutamate-pyruvate transaminase, GPT). Under anaerobic growth conditions, accumulated pyruvate can be utilized by this conversion, conserving carbon which would otherwise be wasted in diffusible ethanol and avoiding cytoplasmic acidosis inherent when high levels of lactate accumulate. This pathway is also important for the assimilation of nitrogen under anoxia or hypoxia. Alanine accumulation can also occur as a result of protein degradation under anoxia. The conversion of pyruvate to Ala does not result in regeneration of NAD^+, which is required for glycolysis to continue. Alanine

aminotransferase also may operate in the opposite direction, utilizing nitrogen from Glu (and ultimately aspartate (Asp), glutamine (Gln) and other amino acids, some of which may be derived from degraded proteins) and producing pyruvate (Xia and Roberts, 1994).

Alanine aminotransferase activity was observed to increase up to fourfold during anaerobic induction in barley (*H. vulgare*) roots (Good and Crosby, 1989). This induction paralleled that of the enzyme ADH. The induction of GPT was observed to be independent of the level of nitrate in the growth solution.

In excised rice roots there was an anaerobic increase in free amino acids derived from protein degradation (Reggiani *et al.*, 1988). The Ala content of hydrolyzed rice root proteins was about 9.5% and Glu, Asp and their amides represented about 18% of the total proteins. Alanine accounted for about 31-32% of the soluble amino acid fraction of anoxic roots and the percentage of free Ala remained fairly constant with the lack of oxygen. This suggested that there was an active interconversion of amino acids released by proteolysis. In 24 hours of anoxia, the alanine derived from proteolysis was only 22.5% of the free Ala accumulated, the remaining 77.5% was a result of amino acid interconversion. In addition, the accumulation of Pro may have resulted via biosynthesis from Glu (Reggiani *et al.*, 1988).

Thomson and co-workers (1989) measured the response of wheat seedlings to low O_2 concentrations in nutrient solutions. They observed that in the excised root tissues exposed to low O_2 concentrations (0.005- 0.01 mol/m^3), there was a net increase in Ala synthesis and an accompanying decrease in the level of free amino acids (not including Ala). Under more severe hypoxia (<0.003 mol/m^3), the free amino acids (excluding Ala) increased by 50%, while Ala increased by 75%. The ratios of carbon in the measured end-products of catabolism between 0.01 and 0.26 mol/m^3 O_2 were 1.7-1.8 in the expanding (apical 6 mm) and the expanded root (10-16 mm from apex) tissues. This indicates that there is either no Pasteur Effect and/or that changes in the pools of amino and organic acids have occurred, in addition to changes in CO_2 evolution. Alanine accumulated both

in the apices of the wheat roots and in the fully expanded tissues at 0.115 mol/m^3 O_2. In the fully expanded tissues, increases in net Ala synthesis under hypoxia were accompanied by either a proportionately smaller increase in the concentration of the other free amino acids or by a net decrease in free amino acids levels (excluding Ala). This supports the view that Ala accumulation is a part of the shift from aerobic to anaerobic catabolism and is not a consequence of decreased protein synthesis. There was a substantial concentration of Ala found in all tissues at 0.115 mol/m^3 O_2, even when compared to ethanol synthesis, indicating that Ala is an important indicator of O_2 deficits in wheat roots (Thomson *et al.*, 1989).

In 1991, Thomson and Greenway reported that in the stele of maize roots exposed to 0.13 and 0.09 mol/m^3 O_2, Ala concentrations were 26% and 44% higher, respectively, than in aerated roots. The increase in stelar Ala concentration was not accompanied by changes in the concentrations of other free amino acids. In the green alga *Selenastrum minutum* there was a rapid drop in Asp and increases in succinate and Ala levels in the first 10 minutes of hypoxia and N-limited growth conditions (Vanlerberghe *et al.*, 1990). The stoichiometry of the changes were close to 1.0 for both moles of Asp lost per mole of Ala accumulated (~1.19, $r^2 = 0.91$) and per mole of succinate accumulated (~0.81, $r^2 = 0.71$). Succinate and Ala levels increased with a stoichiometry of ~1.12 mole of succinate per mole of Ala ($r^2 = 0.76$). After 10 minutes, Asp was depleted and Ala accumulation dropped off to a low rate, while succinate continued to increase.

Alanine and lactate levels were measured using NMR spectroscopy in intact root tips of maize that were hypoxically pretreated (HPT) with 0.3% O_2 and subsequently made anoxic (Xia and Roberts, 1994). They found that the ^{13}C-NMR spectra of the HPT roots were dominated by prominent signals from [3-^{13}C] Ala, in comparison to the non-acclimated (NHPT) roots in which the levels of Ala and lactate were quite similar. During the HPT period, there was an increase in the levels of both lactate and Ala, with a greater increase in lactate. Under anoxia, the increase in Ala is essentially the same in the HPT and NHPT root tips. Therefore, the authors concluded that fermentation to alanine could

not account for the effect of HPT on cytoplasmic pH regulation that was evident in their work.

Lai and co-workers (1992) studied the changes in free amino acids in cell suspension cultures of *M. sativa* under anoxia. They found that the imposition of anoxic conditions caused a dramatic change in the metabolism of the cultured cells. Cytoplasmic acidosis was not observed, instead there was an increase in the pH of the alfalfa cells and the medium. This was explained by the observed increase in the amounts of free amino acids and ammonium in the culture medium. Total free amino acids and the levels of arginine (Arg), tyrosine (Tyr) and lysine (Lys) were measured in the cells and in the B_5 culture medium. Based on two different measurement techniques (ninhydrin and GLC), total free amino acids in the medium of the anaerobic cells increased over the 72 hour period of the experiment, while they declined in the aerobic controls. Of the 19 amino acids detected by chromatography, the changes in Arg, Tyr and Lys predominated. Initially, the concentrations of Arg and Tyr in the culture medium were approximately 10-fold higher than the next most abundant amino acid, indicating that they had been secreted into the culture medium. Anoxia treatment dramatically increased this secretion, especially between 48 and 72 hours. During the same period, the cellular content of these amino acids remained low. Lys accumulation followed a similar pattern of change during anoxia, increasing between 48 and 72 hours in the anaerobic cells, but was not released into the medium.

The increase in free amino acids was correlated with decreased protein contents of the cells under anaerobiosis after 24 hours. The protein content of the medium remained at a low, constant level in the aerobic controls, but increased in the anaerobic treatment with time. The increase of protein in the extracellular medium was not sufficient though, to explain the cellular loss of protein, indicating that substantial proteolysis had occurred (Lai *et al.*, 1992).

In excised rice roots there was also an anaerobic increase in free amino acids derived from protein degradation (Reggiani *et al.*, 1988). Release of amino acids into the growth medium was negligible. The pool of amino acids was separated on a cation exchange HPLC in order to estimate the contribution of each amino acid to the total. Alanine, γ-aminobutyric acid (GABA) and proline (Pro) accumulated with anaerobic treatment. The sum of these three amino acids in anaerobic samples represented about 33% of the soluble amino acid fraction. In contrast, the levels of Glu, Asp and their amides showed a marked decrease during the first hours of anoxia, both in absolute value and as a relative percentage of the total. The ratios of Ala/Glu and GABA/Glu drastically increased and these new ratios were maintained throughout the anaerobic treatment. The levels of the other amino acids were observed to increase during anoxia, but their relative percentages remained more or less unchanged. The shift in the composition of the amino acid pool appeared to be related to changes in the levels of the keto acids; pyruvate, 2-oxoglutarate and succinic acid. Pyruvate and succinic acid increased in the first three hours of anoxia, while 2-oxoglutarate decreased relative to its aerobic level. As the treatment proceeded the levels of the organic acids remained fairly constant, indicating that a new steady state had been reached.

G. Adenylate Energy Charge Ratio

1. Definition and development

The Adenylate Energy Charge (AEC) is a ratio of the energy-storing adenylate nucleotides ATP, ADP and AMP. It is based upon evidence that when the AEC ratio is high, enzymes involved in biosynthetic (anabolic) or other ATP-requiring pathways have higher activities and enzymes involved with regeneration of ATP show reduced activity (Ball and Atkinson, 1975). The AEC ratio has been measured in pure cultures of bacteria, in plant cells and even mycoplasmalike-organisms. Most actively growing cells have AEC values between 0.8 and 0.95, with lower values in anaerobic or starved cells (Salisbury and Ross, 1978).

Considering the importance that energy plays in the metabolic balance of cells, it is not surprising that the availability of metabolic energy (ATP) is a factor in the control of biosynthesis. Sequences such as glycolysis and the citrate cycle, that lead to the regeneration of ATP, are controlled by the energy level of the cell (the balance between ATP, ADP and AMP). The stimulatory effects of AMP on various enzymes in mammals, plants and yeast lends evidence to the theory that the concentrations of the adenine nucleotides are important parameters in the regulation of sequences that lead to the regeneration of ATP or to the production of storage compounds (Atkinson, 1969).

Some enzymes have been shown to respond primarily to the individual concentrations of ATP, ADP, and AMP, whereas others (apparently a larger number), respond to either the ATP/AMP ratio or the ATP/ADP ratio. Because of the heterogeneity of this response, it seemed desirable to devise a single parameter by which the effects exerted on all enzymes by the adenine nucleotides could be related to the energy level of the cell. The relationship:

$$AMP + Pi \Leftrightarrow ATP + H_2O \qquad \text{(Atkinson, 1969)}$$

formally represents energy acceptance and delivery by the adenylate system (Atkinson, 1969). The degree of charge is proportional to the amount of phosphate added to the AMP. On the addition of two moles of phosphate per mole of AMP, the system becomes fully charged and then consists only of ATP. In order that the range of the AEC would be from 0 to 1, rather than 0 to 2, the energy charge ratio was defined as half the number of anhydride-bound phosphate groups per adenine moiety. Thus, it is equal to the mole fraction of ATP plus half the mole fraction of ADP or, in terms of individual concentrations:

$$\text{AEC} = \frac{(\text{ATP}) + 1/2(ADP)}{(ATP) + (ADP) + (AMP)} \qquad \text{(Atkinson, 1969)}$$

On the basis of properties observed *in vitro*, the enzymes of glycolysis and the citrate cycle are tightly controlled by the AEC ratio of the cell. For a cell metabolizing carbohydrate, glycolysis is an amphibolic pathway. It is both catabolic (degrading carbohydrates) and anabolic by supplying, in conjunction with the citrate cycle, most of

the primary metabolic intermediates needed for biosynthetic processes. When considering the control of glycolysis, it is important to consider the latter function of glycolysis. If the pathway was controlled by the AEC ratio alone, glycolysis would be severely limited when the AEC ratio of the cell was high, and this would restrict the supply of acetyl-coenzyme A, keto acids and other starting materials for biosyntheses. This dual control clearly demonstrates the major functions of glycolysis (regeneration of ATP and production of synthetic intermediates) (Atkinson, 1969).

The adenine nucleotides and the AEC ratio can be measured by luminometry. Extracts from firefly lanterns contain the compound luciferin and the enzyme luciferase. Under the influence of ATP and oxygen, the luciferin is oxidized by the luciferase enzyme and part of the energy is released as a single photon of light, enabling quantification of the available ATP using a scintillation counter (Kimmach *et al.*, 1975) or a luminometer. In order to measure the other nucleotides in the equation, a phosphate source, phosphoenol pyruvate and the enzymes PK and myokinase (E.C. 2.7.4.3) are added.

2. Adenine nucleotides in actively metabolizing whole plant tissues

Most plant materials that are used as controls in experiments are not starved of nutrients, and in particular are not limited in the supply of available oxygen. From work with plants and also using data from animal cells (Bomsel and Pradet, 1968), the AEC ratio of non-stressed, actively metabolizing tissues is higher than 0.8 and very often nearer to 0.85 (Bieleski, 1973; Pradet, 1967). For animal cells and microorganisms, the normal AEC ratio lies between 0.85 and 0.94. High AEC ratio values (0.85 to 0.94) were found in germinating seeds of crested wheat grass (Wilson and Harris, 1966), lettuce (Pradet *et al.*, 1968; Raymond and Pradet, 1980), rice (Pradet and Prat, 1976), wheat (Ching and Kronstadt, 1972) and squash (Rasi-Caldogno and DeMichelis, 1978). High AEC ratio values were also observed in wheat (Obendorf and Marcus, 1974) and soybean embryos (Anderson, 1977), in developing wheat grains (Jenner, 1968) and in etiolated coleoptiles of rice (Mocquot *et al.*, 1981). Using the acid extraction method, values

around 0.9 were reported in root tips of maize (Raymond *et al.*, 1987; Saglio and Pradet, 1980; Saglio *et al.*, 1980) and rice (Raymond *et al.*, 1978). In suspension cultured cells of Sycamore (Brown and Short, 1969) and tobacco (Miginiac-Maslow *et al.*, 1981), AEC ratio levels of 0.9 have been measured (using the acid extraction method). Usually, AEC ratio values between 0.7 and 0.8 were found in green leaves of wheat (Bomsel, 1973; Bomsel and Pradet, 1968, Ching *et al.*, 1975; Sellami, 1976; 1977; Sellami and Bomsel, 1975), barley (Luttge and Ball, 1976), winter rape (Sobczyk and Kacperska-Palacz, 1978) spinach (Bonzon *et al.*, 1981) and pinto beans (Weinstein *et al.*, 1969).

In non-green tissues under conditions that limit metabolic activity, the AEC ratio has been observed to be maintained. For example, an inhibition of the respiratory rate (e.g. by limiting the oxygen availability or by inhibiting cytochrome oxidase) automatically produces a lowering of the ATP-requiring processes (Pradet and Raymond, 1983). In lettuce seeds, limitation of the metabolic rate by lowering the temperature induced no observable change in the AEC ratio which remained at values close to 0.9 (Pradet, 1969). Similarly, reducing the metabolic activity in maize root tips by limiting the availability of sugar had no effect on the AEC ratio (Saglio and Pradet, 1980).

On the contrary, conditions such as hypoxia or anoxia limit energy production and simultaneously reduce the respiratory rate and the value of the adenine nucleotide ratios (Pradet and Raymond, 1983). The AEC ratio of rice embryos of seeds in the air increased from a very low value of 0.2 in dry seeds to about 0.9 within one or two hours of imbibition. Under anoxia, the AEC ratio increased more slowly and stabilized around 0.8 after several hours (Pradet and Prat, 1976). When aerobically-grown seedlings were transferred to anaerobic conditions, the AEC ratio stabilized at values between 0.5 and 0.6. It then increased slowly, to stabilize after about 10 hours at 0.8 (Mocquot *et al.*, 1981; Pradet and Prat, 1976). Rice coleoptiles can grow under anaerobic conditions and synthesize DNA, RNA and proteins. This is accompanied by an increase in total adenine nucleotide and an increase in the AEC ratio from 0.6 to 0.8 (Mocquot *et al.*, 1977; Aspart *et al.*, 1980; Mocquot *et al.*, 1981).

Very low AEC ratio values have been found under anoxia in wheat leaves (Sellami and Bomsel, 1975), and in many fatty seeds, including radish (Ali-Ani *et al.*, 1982; Raymond and Pradet, 1980). The AEC ratio was similar when fatty seeds were imbibed under anoxia or imbibed under aeration and then subsequently transferred to anoxia. Fermentative activity and the value of the AEC ratio under anoxia are much lower in fatty seeds than in starchy seeds (Pradet and Raymond, 1983). In excised maize roots, the fermentative capacity can be reduced by depleting the sugar content of the roots by aerobic aging (Saglio *et al.*, 1980). In this material, a correlation between the AEC ratio and the fermentative activity was observed. From these results, it seems that measurement of the AEC ratio in nongreen cells and tissues gives a very good indication of the fermentative activity under anoxia (Pradet and Raymond, 1983).

Under hypoxia, respiratory activity is limited by the availability of oxygen (Pradet and Raymond, 1983). A gradual decrease in the oxygen partial pressure below the critical oxygen pressure (Ducet and Rosenberg, 1962; Pradet and Bomsel, 1978) induces a gradual decrease in adenine nucleotide ratios until the point is reached where the tissue is anoxic (Pradet and Raymond, 1983). This has been observed in lettuce seeds (Pradet, 1969; Raymond and Pradet, 1980; 1982). A correlation between oxygen availability, AEC ratio and protein synthesis was also observed in squash cotyledons isolated from germinating seeds (Rasi-Caldogno and DeMichelis, 1978). It appears that the level of the AEC ratio under hypoxia is clearly correlated with metabolic activity.

H. *Adh* Gene Expression and Regulation

1. The regulation of ADH and the anaerobic response

Evidence of both transcriptional and translational control of the anaerobic response has been identified in a number of plant species. In maize, the patterns of gene expression under anaerobiosis were studied in excised primary roots. The two *Adh* genes; *Adh*1 and *Adh*2, are members of a group of preferentially transcribed genes (Sachs *et al.*,

1980). A common mechanism of gene regulation has been identified in some of the anaerobically induced genes. A maize protoplast transient expression system was used to identify promoter sequences required for anaerobic induction of *Adh*1 (Dennis *et al.*, 1989). The Anaerobic Regulatory Element (ARE), identified in maize cells, contains two subregions, each with a consensus sequence present in all anaerobically regulated genes so far examined (Ricard *et al.*, 1994). Activation of the ARE is thought to occur through sequence-specific transacting DNA-binding proteins. Genomic sequencing, coupled with *in vivo* dimethyl sulfate footprinting was used to identify target sites for binding of the putative transcription factors (Ferl, 1990).

There is also evidence of translational controls of expression occurring during the anaerobic response. Sachs and co-workers (1980) showed that translation of the preexisting set of aerobic mRNA was inhibited and there was a new set of polypeptides synthesized. This appeared to be a result of polysome dissociation (Bailey-Serres and Freeling, 1990). The anaerobic polypeptides (ANPs) accounted for more than 70% of the total proteins synthesized after five hours of anaerobiosis, and their synthesis was maintained in basically the same ratio until the death of the roots (~ 70 hours).

Xie and Wu (1989) showed that the induction of rice *Adh* mRNA correlated with the induction of ADH activity under anaerobiosis. The increase in ADH activity under anaerobiosis was shown to be caused by the accumulation of two ADH polypeptides that were identified by immunoblotting experiments. This accumulation did not depend upon the conversion of inactive proteins to active proteins, but was the result of *de novo* synthesis of the two ADH polypeptides (Kadowaki *et al.*,1988). Therefore, the regulation of ADH enzyme in rice occurs at the transcriptional and/or postranscriptional level (Xie and Wu, 1989).

In soybean seedling roots of the cultivar 'Williams', Russell and co-workers (1990) found a linear relationship between ADH specific activity and duration of anoxia. *In vivo* protein labeling followed by nondenaturing-SDS polyacrylamide gel

electrophoresis yielded a simple pattern, with only four spots being resolved. One of the spots comigrated with ADH in the native dimension and was estimated to have a molecular weight about 40 kD in the SDS-denaturing dimension. Northern blot analysis showed that *Adh* mRNA levels increased during the first hours of anaerobiosis, then started to decrease by about 12 hours.

2. Isozymes of ADH

The term 'isozymes' or 'isoenzymes' was coined by Market and Moller (1959) to describe different molecular forms of proteins with the same enzymatic specificity. The two terms have the same meaning and are used interchangeably. Isozyme variation may arise in a number of ways. It may be the result of multiple, structurally different genetic loci (genic isozymes) or from differences in unlike alleles at a single genetic locus (allelozymes or allozymes) (Moss, 1982). Expression of isozymes may vary in different plant tissues, at different developmental stages of the plants' life and under stress conditions.

Maize *Adh*1 is probably the most intensely studied gene involved in the anaerobic response in plants (Freeling and Bennett, 1985). *Adh*1 encodes the major ADH enzyme and is expressed in the seed, seedling and pollen of maize, but not in the leaves. The active enzyme is a dimer of 80 kD in maize. In maize seedlings subjected to anaerobiosis, there are three electrophoretically separable genic isozymes of ADH induced in the roots (Freeling and Schwartz, 1973). These three isozymes are formed by the random dimerization of the polypeptides encoded by two different *Adh* genes, *Adh*1 and *Adh*2. Set 1 enzymes ($ADH_{1/1}$) migrate most slowly towards the anode; Set III ($ADH_{2/2}$) isozymes migrate the fastest, and Set II ($ADH_{1/2}$) isozymes move at an intermediate rate.

Among higher plants, rice (*O. sativa*) is unusual in that its seed can germinate in an environment of extremely low oxygen content, even under a vacuum (Cobb and Kennedy, 1987). Anaerobic treatment of rice seedlings increased the specific activity of

ADH in embryos, endosperm, roots, etiolated leaves and mature green leaves (Xie and Wu, 1989). In comparison to maize, which lacks any ADH activity in the mature green leaves, the expression of ADH in the leaves of rice is unique and is probably an adaptation (Okimoto *et al.*, 1980; Freeling and Bennett, 1985).

Rice ADH was observed to be induced by 2,4-dichlorophenoxyacetic acid (2,4-D) under aerobic conditions, as in maize (Freeling, 1973), but to a lower level than that caused by anaerobiosis (Xie and Wu, 1989). Starch gel electrophoresis of protein extracts from rice showed three distinct isozymes of ADH, two minor ones ($ADH_{1/2}$ and $ADH_{2/2}$) and one major one ($ADH_{1/1}$). These enzymes were determined to be dimers of two 40 kD subunits encoded by separate genes; *Adh*1 and *Adh*2. The expression of the ADH isozymes varied with the organ, suggesting that the expression of ADH genes in rice is organ-specific. In roots, $ADH_{2/2}$ was predominantly expressed, while $ADH_{1/1}$ enzymes were expressed in the leaves, sheaths, and pollen (Xie and Wu, 1989).

There is also evidence of developmental control of ADH expression in rice. During seed development, all three isozymes are expressed (Xie and Wu, 1989). The $ADH_{2/2}$ form was detected very early in seed formation (grain-filling stage), although it was detected at much lower levels than the other two. At the later stages of seed development (ripening stage and mature dry seed), $ADH_{2/2}$ and $ADH_{1/2}$ bands gradually disappeared, suggesting that the activities of these enzymes are selectively lost during grain maturation. In mature dry seeds, $ADH_{1/1}$ is the predominant isozyme expressed.

After germinating in air for three hours, the homodimer of $ADH_{1/1}$ was the still the most prevalent isozyme expressed, although there was another fainter band, anodic to the first. In rice embryos germinated for 48 hours in the air, then submitted to anoxia for at least six hours, a second anodic band appeared. The intensity of the two anodic bands increased as time of anoxic treatment increased (Ricard *et al.*, 1986).

Three genes specify alcohol dehydrogenase in barley (*H. vulgare*) (*Adh*1, *Adh*2 and *Adh*3) (Hanson *et al.*, 1984). Their polypeptide products (ADH_1, ADH_2, ADH_3)

dimerize to give a total of six ADH isozymes that can be resolved by native gel electrophoresis and stained for enzyme activity. Good and Crosby (1989) observed that N_2-treated seedling roots of barley had six bands of ADH activity, compared to the air-treated roots, which had only one. Dry seed showed a strong accumulation of a single band, present also in the seedling roots. This band was absent from the seed and seedling roots of an ADH1$^-$ mutant. Earlier work (Hanson *et al.*, 1984), had shown that the constitutive ADH activity of barley seeds was primarily $ADH_{1/1}$ homodimer, which accumulates during seed development.

In aleurone layers of barley (*H. vulgare* cv. Himalaya), there was a high titer of a single isozyme, the homodimer of $ADH_{1/1}$, under aerobic conditions. This isozyme was accumulated by the aleurone tissue during the later part of seed development and survived seed drying and rehydration. The five other ADH isozymes were induced by O_2 deficit and their staining intensity increased progressively as O_2 levels were reduced below 5%. In *H. spontaneum*, as seen in *H. vulgare*, the *Adh*1 locus is constitutively expressed in seed tissues, whereas expression of the loci *Adh*2 and *Adh*3 requires anaerobic induction (Hanson and Brown, 1984).

In 1986, Brzezinski and co-workers reported that in the soybean (*G. max*), which has two ADH genes, ADH was not inducible by flooding, although it was induced as much as 14-fold by 2,4-D. They found that soybean radicle tissue was rich in ADH, but enzyme activity decreased slowly with the development of roots and was almost undetectable by the time the first true leaves appeared. A single ADH activity band was resolved in anaerobically-treated seedling roots (Russell *et al.*, 1990; Newman and VanToai, 1991; 1992), radicle tissue (Brzezinski *et al.*, 1986), leaves and hypocotyls (Newman and VanToai, 1991). Kimmerer (1987) observed that ADH activity increased in soybean embryos upon imbibition, then declined exponentially with development. The cotyledons of unstressed soybean seedlings contained three isozymes, two were distinct and one was faintly visible. All three of the isozymes increased in activity upon submergence. Anoxia treatment increased the intensity of staining of the bands, but did

not result in changes in the zymogram patterns. This pattern fits a model of a two gene system, where both genes are expressed in the cotyledons and only one is expressed in the roots, leaves and other tissues (Newman and VanToai, 1991).

Three equally spaced isozymes were detected in developing and mature (imbibed) seeds of peas (*P. sativum* cv. Greenfeast). The same three isozymes were present in the roots and epicotyls of young seedlings, but at considerably lower levels. With anaerobic induction, the activity of all three isozymes in the cotyledons, roots and epicotyls of the seedlings increased three to four-fold. The maximum activity was reached after about 12 hours, but declined by 24 hours as the seedlings began to deteriorate. An additional three isozymes, not detected in the mature seeds were present in the immature cotyledons. These developmentally-specific isozymes reached their maximum between seven and ten days post-pollination (Llewellyn *et al.*, 1987).

Allelic differences in isozymes are thought to arise from mutations in unlike alleles at the same locus. These variant alleles may occur with frequencies similar to those of the most usual variant, in which case the population is said to be polymorphic with respect to the isozymes (Moss, 1982). Different cultivars of maize carry *Adh*1 alleles coding for subunits that make up different ADH_1 isozymes, most of which confer different electrophoretic mobilities to the dimeric enzyme containing these subunits (Schwartz and Endo, 1966). Maize is polymorphic for Set I ($ADH_{1/1}$) and Set II ($ADH_{1/2}$) isozymes as a result of the naturally-occurring variant alleles present at *Adh1*. No polymorphism has been reported in Set III ($ADH_{2/2}$) (Freeling and Bennett, 1985).

Two *Adh1* alleles were chosen from commercial US cultivars as references and are designated *Adh1-1*S and *Adh1-1F*. Heterozygotes were constructed where the relative expression of each allele could be compared in different organs of the plant by quantification of the allozyme activity or protein ratios (Freeling and Bennett, 1985). The variant alleles that are relatively over-expressed in the scutellum are relatively under-expressed in the root and vice versa. This organ-specific "reciprocal effect" is at the

polypeptide synthesis level or before. In addition to organ specificity, the alleles differ in subunit surface charge, but not in specific activity at pH 8.0 (Schwartz, 1973). Sequence differences in the variant alleles were identified by S_1-nuclease analyses using root mRNA (Dennis *et al.*, 1984) and it was found that there were 14 substitutions in 1134 bp of translated sequence, including two that that encode different amino acids.

Polymorphisms in the *Adh*3 locus were observed in N_2 -treated embryos and aleurone tissues of barley's wild progenitor; *H. spontaneum* (Hanson and Brown, 1984). After a fairly small-scale (*sic*) survey of *H. spontaneum* lines, the authors found a total of five alleles for the *Adh*3 locus, including an *Adh*3n variant that lacked the immunologically active protein product (Hanson and Brown, 1984).

Three electrophoretic variants of ADH isozymes were found in *A. thaliana* (which has only one *Adh* gene), based upon their mobility on Tris-citrate starch gels (Dolferus and Jacobs, 1984). These variants were identified from callus material of 65 geographical races. The electrophoretic variants were designated S (slow), F (fast) and A (superfast). From crosses made between the races with the ADH variant isozymes, it was concluded that the variation in ADH was controlled by three alleles at a single genetic locus (*Adh*1-S, *Adh*1-F and *Adh*1-A) with codominant expression. *In vitro* dissociation-reassociation experiments determined that the ADH enzyme in *A. thaliana* was a dimer and the molecular weight of the native enzyme was estimated to be 87,000 by gel filtration using marker proteins. The molecular weight of the subunits was found to be 45 kD for all three of the electrophoretic variants.

There was a low level of ADH activity in soaked seeds. Upon germination, the activity declined rapidly and disappeared completely with the development of the first leaves. Zymograms of different plant organs; seeds, cotyledons, roots, and callus showed the same isozyme, staining most intensely in the callus material. Alcohol dehydrogenase activity was also demonstrated by histochemical staining in pollen grains, around the

vascular bundles from leaves and stems, and in the meristematic region of the root apex (Dolferus and Jacobs, 1984).

2. Analysis of *Adh* Mutants

An efficient system was developed to obtain *Adh*1 mutants in maize (Freeling and Bennett, 1985). Quiescent seeds, homozygous for a *Adh*1 allele which specified an $ADH_{1/1}$ allozyme of a particular electrophoretic mobility (for example F or S), were treated with ethyl methanesulphate (EMS). Selected plants from the mutagenized seed were crossed to pollen from an *Adh*1 tester line which carried an *Adh*1 allele with a different electrophoretic mobility. Sectors heterozygous for an *Adh*1 mutant were recognized by deviations from normal allozyme ratios quantified following electrophoretic separation in starch gels. This procedure allowed the scoring of almost all sorts of transmissible ADH1 mutants, including those causing changes in specific activity, charge, dimerization and timing of expression.

*Adh*1 null mutants were selected from the population of the mutagenized plants using allyl alcohol vapours (Freeling and Bennett, 1985). In the presence of ADH, allyl alcohol is oxidized to a poisonous acrolein. Haploid pollen grains that survived this treatment were functionally ADH^-, since $ADH_{1/1}$ was the only ADH activity in male gametophyte. The ADH^- pollen can be used in the crosses described above. Plants homozygous for ADH^- in the roots were unable to survive longer than a few hours flooding. Root meristems made lactic acid continuously and apparently died as a result of cytoplasmic acidosis, and not because of energy starvation as a result of pH inhibition of glycolysis (Roberts *et al.*, 1984a; 1984b).

A barley mutant, *Adh*1-M9 has been identified that lacked a functional ADH1 gene product (Harbred and Edwards, 1982). The mutant allele, when homozygous, resulted in the absence of Set I ($ADH_{1/1}$) and Set II ($ADH_{1/2}$) isozymes and presumably either failed to code for an ADH_1 polypeptide or coded one that was both enzymatically inactive and incapable of normal dimerization. In comparison to $Adh1^+$ seeds, the *Adh*1-M9 seeds were extremely sensitive to immersion. The viability of the $Adh1^+$ seeds

decreased slowly over the course of a few days, while that of the *Adh*1-M9 fell sharply within a few hours following the onset of immersion. Clearly there is a specific requirement for ADH activity in the barley seeds, if they were to survive even a short period of time immersed (Harbred and Edwards, 1982). Seedlings grown in a flooded situation did not show a significant difference in root growth or survival between the *Adh*1-M9 and the *Adh*1^{+}, although the mutant line contained approximately 50% of the ADH1 of the wild-type roots (Harbred and Edwards, 1982).

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Chapter II. The Hypoxic Response in Seedling Roots

Introduction

The supply of oxygen to a seedling root depends upon the concentration of the gas as well as its rate of diffusion through the medium to the site of utilization. Seedlings that are flooded are susceptible to both root anoxia and hypoxia since the diffusion of oxygen is about 100 times slower in water than in air (Thomson and Greenway, 1991). Hypoxic metabolism is characterized by the concurrent activity of both limited respiration (aerobic metabolism) and some degree of fermentation (anaerobic metabolism) (Ricard *et al.*, 1994). When hypoxia reduces respiration to 5% of its initial value, the oxidative production of ATP is, in the absence of the Pasteur Effect, equivalent to that produced by fermentative processes (Pradet and Bomsel, 1978).

One strategy for survival is the avoidance or amelioration of the stress by the formation of adventitious roots and aerenchyma tissue, which increases the diffusion of oxygen from aerated portions of the plant to the roots. The formation of lysigenous or schizogenous aerenchyma, which is believed to be mediated by ethylene accumulation, improves the internal movement of oxygen to the root apex (Jackson, 1985). This has been observed in many species under hypoxic or anoxic conditions (Drew *et al.*, 1979; 1980; 1985; Saglio *et al.*, 1983; Jackson, 1985).

To survive anoxia or hypoxia stress, the plant must maintain the Adenylate Energy Charge (AEC) ratio. This may depend upon several factors, for example, adequate supplies of carbohydrates (Saglio *et al.*, 1980; Barclay and Crawford, 1982, Braendle and Crawford, 1987), an acceleration of glycolysis and fermentation (Raymond *et al.*, 1985; Cobb and Kennedy, 1987) and/or the down-regulation of non-essential metabolic pathways to conserve the energy that is produced (Sachs *et al.*, 1980).

Conditions such as hypoxia or anoxia limit energy production and simultaneously reduce the respiratory rate and the value of the adenine nucleotide (AdN) ratios (Pradet

and Raymond, 1983). For example, when aerobically-grown rice seedlings are transferred to anaerobic conditions, the AEC stabilized at values between 0.5 and 0.6. It then increases slowly, to stabilize after about 10 hours at 0.8 (Mocquot *et al.*, 1981; Pradet and Prat, 1976). Under these conditions rice coleoptiles synthesize DNA, RNA and proteins and continue to elongate. This is accompanied by an increase in total AdNs and an increase in the AEC from 0.6 to 0.8 (Mocquot *et al.*, 1977; Aspart *et al.*, 1980; Mocquot *et al.*, 1981).

The objectives of these experiments were to determine how *Brassica napus* seedlings would respond to hypoxic root conditions. I hypothesized that the levels of ADH enzyme activity would increase under hypoxia and that these changes would be consistent with changes in the quantity of ADH polypeptide observed. I also wanted to determine if there were temporal changes in the ADH activity and ADH isozymes under hypoxia. As well, I wanted to examine whether aerenchyma tissue would occur in adventitious roots of seedlings under long-term hypoxia. Finally, I wanted to characterize the changes that occur in the AEC ratio and the level of TAs during hypoxia treatment.

Materials and Methods

Plant Growth Conditions

Plants were grown in 20 cm pots in a peat:sand:vermiculite (2:1:2) mixture in an environmental chamber (250 μmol m^{-2} s^{-1}, 16 hour photoperiod, 20°C/15°C (light/dark)) for four to five weeks. Upon the onset of flowering, the plants were transferred to 10°C/7 °C under the same light and photoperiod regime to continue growth. The plants were fertilized twice weekly using a dilute solution of complete 20:20:20 fertilizer (Plant Prod., Plant Products Co. Ltd.).

The original seed (*Brassica napus*, R8311) was obtained from Dr. Rachael Scarth, Department of Plant Science, University of Manitoba, Winnipeg, Manitoba. Plants were

self-pollinated for the purpose of seed increase. This line was chosen because of its utility for microspore-derived haploid embryo production. The line R8311 was derived from the cultivar Ariel (Dr. Rachael Scarth, personal communication).

Self-Pollination of Plants for Seed Production

Plants used for seed production were grown in an environmental chamber (250 μmol m^{-2} s^{-1}, 16 hour photoperiod, 20°C/15°C (light/dark)). Before bagging the inflorescence, any open flowers were removed to prevent the development of outcrossed progeny. Each inflorescence was covered with a cellophane, micro-wicketted, bag (11 cm by 20 cm) (Cryovac Division, W.R. Grace and Co. of Canada Ltd.). Once the siliques had matured, the watering regime was reduced as the seed dried. The seed was collected, cleaned by hand and stored in an airtight bag at room temperature.

Seedling Treatment in the Hydroponic Treatment System

Seeds were surface sterilized in 1.2% sodium hypochlorite (with two drops of Tween-20 as a surfactant) for 20 minutes, followed by three rinses (five minutes each) in sterile, deionized water. The seeds were germinated in glass petri plates containing sterilized perlite, in an environmental chamber (120 μmol m^{-2} s^{-1}, 16 hour photoperiod, 22° C). The perlite was maintained moist, but not wet, by a filter paper wick in a tray of half-strength Hoagland's solution (Wetter and Constabel 1982). By day three the cotyledons had emerged from the seed coat and greened, commencing autotrophic growth. Greened seedlings were selected at the cotyledonary stage, at the time of hypocotyl hook straightening (three to four days old). Seedlings were individually inserted into foam plugs and placed into Styrofoam rafts floating in half-strength Hoagland's solution in rectangular, polyethylene containers (4.0 L, 14 cm by 40 cm). The ends of the Styrofoam rafts were covered with black plastic. Treatment gas was supplied to each container through a polyvinyl chloride tubing line (Nalgene Co., Fisher Scientific Ltd.) from the gas cylinder, through a small hole, to a 15 cm aerator stone in the nutrient solution. The flow rate was 350 mLs per minute, as measured using a Fischer and Porter Flow Meter. The roots of the seedlings were suspended in the nutrient solution, which was sparged

continuously with either nitrogen gas (extra dry, minimum purity: 99.95% N_2, Linde, Union Carbide) or air (extra dry, Linde, Union Carbide), to create either hypoxic or well-aerated growth conditions, respectively. The dissolved oxygen content in the nutrient media (as measured using a YSI Oxygen meter, Model 54A) was 10.8-11.2 mg O_2/L (28%-29% O_2) in the air treatment and 2.5-2.9 mg O_2/L (6.4%-7.5%) in the hypoxic treatment. The control seedlings were left in petri plates containing sterilized perlite and were wick-watered with half-strength Hoagland's solution. Both the seedlings in the Hydroponic Treatment System and the control seedlings in the petri plates were placed in an environmental chamber (120 *u*mol m^{-2} s^{-1}, 16 hr photoperiod, 22°C).

Fresh and Dry Weight Measurements

Seeds were germinated (as above) and either treated in the Hydroponic Treatment System or left in petri plates of perlite (as above). After the appropriate time period, the seedlings were sectioned into roots and shoots (hypocotyl and cotyledons). After the fresh weights were measured, the seedling sections were dried for 18 to 24 hours at 80°C under vacuum, then reweighed. For each data point, three independent experiments were performed, consisting of two samples of five seedlings each (n=3). The standard error of the mean was calculated to illustrate the variation in the samples.

ADH Enzyme Assays

Alcohol dehydrogenase (ADH) enzyme activity was determined spectrophotometrically by a modification of the methods of Wignarajah and Greenway (1976), Dolferus and co-workers (1985) and Good and Crosby (1989). Seedlings were removed from the Hydroponic Treatment System, and the roots were quickly rinsed in deionized water, blotted dry, and excised. The roots were ground in 500 *u*L grind buffer (100 mM Tris-HCl (pH 7.5), 10 mM dithiothreitol, 10 mM sodium borate, and 15% v/v glycerol) in 1.5 mL microfuge tubes using a Pellet Pestle (Kontes). The crude homogenates were microcentrifuged for five minutes at 4°C at 13,000 rpm (~ 6000 x g). Aliquots were removed from the supernatant for the enzyme assay. The sample aliquot was added to the enzyme assay mixture (grind buffer containing 89 mM acetaldehyde and

NADH (final concentration 100 *u*M)) to a total volume of 500 *u*L, mixed well by inversion, and placed into the spectrophotometer which had been blanked before measuring the activity. The oxidation of NADH to NAD was measured as a decrease in absorbance at 340 nm. The ADH activity of the samples was normalized on the basis of the protein content (*u*mol NADH /mg protein/minute) using either Bradford's (Bradford, 1976) or Peterson's method of protein determination (Peterson, 1977), a modification of the Lowry (Lowry *et al.*, 1951) method. The rate of change of the absorbance at 340 nm was shown to be directly proportional to the amount of the sample aliquot. All enzyme assays were performed at 25°C. Each data point was the mean of three independent experiments, consisting of two samples of five seedlings each (n=3). The standard error of the mean was calculated. An Analysis of Variance (Anova) was performed to determine if the variation between the treatments was significant (see Appendix).

Western Blots

Seedlings were removed from the Hydroponic Treatment System, and the roots were ground immediately in ADH assay grind buffer, as above. The ground samples were microcentrifuged at 4°C for 5 minutes at 13,000 rpm (~ 6000 x g). The soluble protein content was determined in an aliquot of the supernatant using the method of Peterson (1977). An aliquot of the supernatant was diluted 1:3 in SDS sample buffer (62 mM Tris-HCl, pH 6.8, 10% glycerol (v/v), 2% sodium dodecyl sulphate (SDS) (w/v), 5% 2-β-mercaptoethanol and bromophenol blue as a tracking dye). The diluted aliquots were boiled for five minutes and cooled on ice. An equal amount of total buffer-soluble protein (30 *u*g (Figure 2-4a) and 10 *u*g (Figure 2-4b)) was separated in Laemmli running buffer (25 mM Tris-190 mM glycine (pH 8.5)) (Laemmli, 1970) on a 1.5 mm thick, 12% SDS polyacrylamide gel (11.68% acrylamide- 0.32% N, N`-methylene bisacrylamide (bis), with 0.1% SDS and 0.375 M Tris-HCl (pH 8.8)). A 4% stacking gel (3.8% acrylamide- 0.2% bis, with 0.1% SDS and 0.125 M Tris-HCl (pH 6.8)) was used. The gels were polymerized with 0.08% (v/v) N, N, N`, N`-tetramethylene ethylene diamine (TEMED) and 0.05% ammonium persulfate (APS). The gels were run at 180 volts (constant voltage) using either a Bio-Rad large gel apparatus (Figure 2-4a) or a Bio-Rad Minigel

apparatus (Figure 2-4b). The proteins were electroblotted onto supported nitrocellulose membranes (Schleicher and Schuell, Keene, N.H.) in 10 mM sodium bicarbonate and 3 mM sodium carbonate in 20% methanol (pH 9.8) (Dunn, 1986) at 75 volts (constant voltage) for two hours at 4°C. The blots were stained in Ponceau-S to check for transfer efficiency (Salinovich and Montelaro, 1986). The nitrocellulose membranes were blocked for 2.5 hours at 4°C in Blot Rinse Buffer (BRB) (10 mM Tris-HCl (pH 7.4), 150 mM sodium chloride, 1 mM EDTA with 0.1% thimerosal and 0.1% Tween) with 5% Bovine Serum Albumin (BSA). The membranes were incubated for 2.5 hours at 22°C in primary ADH antibody (1:1000 dilution in BRB with 5% BSA), then washed three times for 10 minutes each in BRB. The nitrocellulose membranes were incubated for two hours at 22° C in a 1:3000 dilution of goat anti-rabbit IgG (H+L) Horseradish Peroxidase Conjugate (Bio-Rad), followed by two washes of ten minutes each in BRB and two washes in Tris-buffered saline solution (TBS) (10 mM Tris-HCl (pH 7.4), 150 mM sodium chloride). The blots were developed in TBS with 2.8m M 4-chloro-1-napthol dissolved in 16.7% methanol and 0.015% hydrogen peroxide. The size of the ADH subunit was determined by comparison with Bio-Rad Low Molecular Weight Standards (14.4 kD to 97 kD).

The ADH polyclonal antibody was kindly provided by Dr. Allen Good, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada. It was prepared against purified ADH protein from barley (Good and Crosby, 1989). Three indendent western blots were analyzed using a Bio-Rad Imaging Densitometer, Model GS-670 and Bio-Rad Molecular Analyst software, version 2.1. All densitometer scans were within the linear range of the instrument.

Nondenaturing PAGE of ADH isozymes

Non-denaturing polyacrylamide gel electrophoresis (PAGE) for isozymes of ADH was performed according to Hanson and Jacobsen (1984). Root samples were ground in 500 *u*L ADH assay buffer as above, in 1.5 mL microfuge tubes using a Pellet Pestle (Kontes). The crude homogenates were microcentrifuged for five minutes at 4°C at 13,000 rpm (~ 6000 x g). The soluble protein content was determined in an aliquot of the

supernatant using the method of Peterson (1977). Aliquots containing 25 *u*g of buffer-soluble protein (with 15% glycerol and 0.01% bromophenol blue) were run on a 1.5 mm, 7.5% nondenaturing polyacrylamide gel (7.4% acrylamide- 0.1% (bis), with 0.38 M Tris-HCl (pH 8.5)). The gel was polymerized with 0.08% v/v TEMED and 0.05% APS and run at 4°C, in Laemmli running buffer (Laemmli, 1970) at 75 volts. The gels were stained in a solution containing 7.6% ethanol, 452.2 *u*M nicotinamide adenine dinucleotide (NAD), 244.6 *u*M nitroblue tetrazolium chloride (NBT) and 163.2 *u*M phenazine methosulphate (PMS) in 0.1 M Tris-HCl (pH 8.0). The staining was performed at room temperature until blue-black ADH bands had appeared.

Fixing, Embedding and Sectioning of Root Tissue

Seeds were germinated and treated in the Hydroponic Treatment System for seven days, as above. The primary roots and adventitious roots were rinsed in deionized water, blotted dry and cut into 1 cm sections. The root sections were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) by vacuum infiltration overnight. The fixed root sections were washed in 0.1 M phosphate buffer (pH 7.0) three times, for 15 minutes each. Ethanol dehydration was done in 15% increments, starting at 15%, for 30 minutes each (15%, 30%, 45%, 60%, 75%, 90%). After three 30 minute washes in 95% ethanol, the root sections were transferred to propylene oxide in three graded steps of ethanol:propylene oxide (3:1, 1:1, 1:3 ratios) for 15 minutes each, followed by three changes of 100% propylene oxide. Infiltration with Spurr's resin (Spurr, 1969) was done in two graded steps of propylene oxide: Spurr's (1:1, 1:2 ratios) for three hours each. Any residual propylene oxide was evaporated overnight in the final infiltration step of pure Spurr's resin. After two transfers to Spurr's resin for three hours each, the root sections were embedded in Spurr's resin in rubber molds. The resin blocks were cured at 70°C overnight and cooled at room temperature for 24 hours. The Spurr's resin was prepared by mixing vinyl cyclohexene dioxide (ERL 4206), diglycidyl ether of polypropylene glycol (DER 736), nonenyl succinic anhydride (NSA) and dimethyl amino ethanol (S-1) (all from J.B. EM Services, Dorval, Quebec) according to Spurr (1969). The solution was stirred thoroughly and stored at -20°C in 10 mL disposable plastic syringes.

Before sectioning, the resin blocks were trimmed using a hand Dremel (Emerson Electric Co., Racine, WI) and fine sandpaper. The blocks were mounted into a Reichert Om U2 Ultramicrotome fitted with a glass knife. The sections (1 *u*m each) were transferred to glass slides prepared in the following manner. The slides were washed with 95% ethanol for one minute, then dipped into a 5% gelatin solution (with 0.5% chromium potassium sulphate) for 30 seconds, then allowed to dry and stored in a covered container. The sections were fixed to the slides by warming the slides on a hot plate at a low temperature setting. The sections were stained for 30 seconds using 1% toluidine blue in 1% sodium borate solution and the excess rinsed off with distilled, deionized water. The sections were photographed using an BH-2 microscope with an Olympus C-35AD-4 camera attachment and Olympus Exposure Control Unit using Kodak Technical Pan film and printed on Kodabrome II RC paper.

Determination of Adenylates

Seedlings were removed from the Hydroponic Treatment System and the roots were quickly rinsed in deionized water, blotted and excised. They were ground immediately in 250 *u*L 2.5% trichloroacetic acid (TCA) with 2 mM ethylenediamine tetraacetic acid (EDTA) in 1.5 mL microcentrifuge tubes using a Pellet Pestle (Kontes) and held on ice for 30 minutes. The homogenates were microcentrifuged at 4°C for 5 minutes at 13,000 rpm (~ 6000 x g). A 100 *u*L aliquot of the supernatant was diluted 250 times with ice-cold 100 mM Tris-acetate (Tris-Ac) (pH 7.75) with 2 mM EDTA. Aliquots of the diluted extract were assayed for the three adenine nucleotide phosphates by a modification (Pomeroy and Andrews, 1986) of the method of Pradet (1967). In parallel determinations on the same samples, the adenine nucleotides (AdN) were measured using the firefly luciferin/luciferase system (LKB-Wallac Bioluminesence ATP Monitoring Reagent). The ATP in the sample was measured in a 100 *u*L aliquot of the diluted extract that was incubated for 30 minutes at 22°C with 100 *u*L of Buffer A (20 mM Tris-Ac (pH 7.75) with 7.5 mM magnesium acetate and 80 mM potassium acetate. The adenosine diphosphate (ADP) in the samples was converted to ATP for measurement

by incubating a 100 *u*L aliquot of the diluted extract in 100 *u*L of Buffer B (Buffer A with 4 units of pyruvate kinase (PK; E.C. 2.7.1.40 and 25 nM phosphoenol pyruvate). Similarly, adenosine monophosphate (AMP) was converted to ADP and subsequently to ATP by incubating a 100 *u*L aliquot of the diluted extract with 100 *u*L of Buffer C (Buffer B with 12.5 units myokinase). After the incubation period, an 100 *u*L aliquot of the incubation mixture was combined with 300 *u*L of Reaction Media (100 mM Tris-Ac, pH 7.75) and 100 *u*L of LKB-Wallac Bioluminesence ATP Monitoring Reagent (Fisher Scientific). Luminescence was recorded on LKB 1250 and 1251 Luminometers (LKB-Wallac, Turku, Finland) and the response rate in each sample was recorded from three successive additions of 1 *u*M ATP in a volume of 10 *u*L, to give a final concentration of 32.3 nM ATP. The Adenylate Energy Charge was calculated by the method of Atkinson (1969).

$$\text{AEC} = \frac{(\text{ATP}) + 1/2(ADP)}{(ATP) + (ADP) + (AMP)}$$

The Total Adenylate values were normalized between samples on the basis of total solubilized protein as determined by the procedure of Peterson (1977). All the enzymes and chemicals were obtained through Sigma Chemical Company, with the exception of the Bioluminesence ATP Monitoring Reagent (Fisher Scientific). For each data point three independent experiments (of three samples of five seedlings each) were performed (n= 3). The standard error of the mean was calculated. An Analysis of Variance (Anova) was performed to determine if the variation between the treatments was significant (see Appendix).

Results and Discussion

Survival of seedlings during periods of flooding depends upon their ability to compensate for hypoxic growth conditions and maintain critical metabolic processes. The goal of this study was to determine how seedling roots of *Brassica napus* would respond to hypoxic treatment. It was hypothesized that hypoxic conditions would result in increases in the level of ADH enzyme activity and maintenance of the AEC and total

adenylate levels over the treatment period. I also wanted to determine if there were temporal changes in the ADH activity and ADH isozymes under hypoxia.

Since anoxic or hypoxic growth conditions in the root environment limit respiration, one of the most prevalent effects is an inhibition of normal growth. Figure 2-1a shows the Hydroponic Treatment System used to hypoxically treat the seedling roots. In Figure 2-1b, the effects of the hypoxia treatment on shoot and root growth are shown. Reduced growth under anoxia or hypoxia has been shown in seedlings of barley (Wignarajah *et al.*, 1976), rice (Wignarajah *et al.*, 1976; Cobb and Kennedy, 1987), wheat (Thomson *et al.*, 1989; Huang *et al.*, 1994) and *Echinochloa oryzicola* (Cobb and Kennedy, 1987). Reduced yields of oilseed rape (*B. napus*) (Cannell and Belford, 1980) have also been shown.

Figure 2-2 shows how the average seedling fresh and dry weights (Figures 2-2a and 2-2b) change over a seven day period of treatment with either air or nitrogen gas, in comparison to control seedlings. By day three, differences in fresh weight and dry weights could be seen between the N_2 and air-treated seedlings. The higher growth rate in the air-treated seedlings became more apparent between three and seven days treatment. There was little difference between the fresh weights of the control seedlings and that of the N_2-treated seedlings, but the dry weights of the control seedlings were slightly higher than those of the N_2-treated ones after three days. This may indicate that the N_2-treated seedlings had a higher proportion of water than did the control ones. As well, this may indicate that the control seedlings were slightly hypoxic.

Generally, the adverse effects of flooding on seedling growth are more evident in the roots than in the shoots. This has been observed in seedlings of wheat (Thomson *et al.*, 1989; Huang *et al.*, 1994) and barley (Wignarajah *et al.*, 1976). Results from the present study show that there is a slightly greater effect on the shoots than the roots. After seven days, there is approximately 43% difference in the fresh weight between the air and N_2-treated shoots, while in the roots there is a difference of approximately 28%.

When the fresh weights of the shoots and roots are examined after three days growth, this difference is much smaller, with approximately 30% difference in the shoots and only 23% in the roots. This difference is also seen in the dry weight data, with differences after seven days of 40% in the shoots and only 31% in the roots.

The reason for this discrepancy from the published work may be a difference in the sampling times used or a result of the differences between the growth of monocots and dicots. Root and shoot growth was measured in wheat (Huang *et al.*,1994) after 14 days treatment and in barley (Wignarajah *et al.*, 1976) after 12 days. Rice, a flood-tolerant species, does not demonstrate an inhibition of root growth under hypoxia (Wignarajah *et al.*, 1976). When seedlings of rice or *Echinchloa crusgalli* var. *oryzicola* (a flood-tolerant weed), were treated with anoxia, root growth, and to a lesser extent, shoot growth was inhibited, but resumed upon the return to well-aerated conditions (Cobb and Kennedy, 1987).

Alcohol dehydrogenase enzyme activity was measured daily in the roots of seedlings over a seven day treatment period (Figure 2-3). The seedlings were approximately four days old at the beginning of the treatment period (at the stage of hypocotyl hook opening). Under hypoxic growth conditions, the level of ADH activity increased significantly over that in the roots of the air-treated seedlings (see Anova in Appendix). The level of ADH activity in the roots of the control seedlings was intermediate between that of the other two treatments. It is possible that the aeration treatment was suppressing the ADH activity. The concentration of dissolved O_2 in the air treatments in the Hydroponic Treatment System was approximately 28% to 29%. A three-fold decrease in ADH activity was observed when maize root tips were treated with 40% O_2 (Johnson *et al.*, 1989) and a five-fold decrease was seen in rice and barley roots treated with 20% O_2 (Wignarajah *et al.*, 1976).

In all three treatments, there is a biphasic pattern evident in the ADH activity over the seven day treatment period, with an initial increase before day one and a second

increase that occurred after a slight drop in activity around day three. This is especially evident in the N_2-treated seedling roots, and to a lesser extent, in the air-treated ones. This is unlikely to be a treatment effect since the same trend is also seen in the control seedlings. It appears as though the level of ADH activity or its inducibility changes with time in this system. A somewhat similar pattern of ADH activity was seen in cell suspension cultures of alfalfa (*Medicago sativa*) under anoxia stress. In the anaerobic suspension cells, ADH activity rose rapidly during the first six hours, then fell within 12 hours to a level below that at time zero. Between 12 and 24 hours, the activity rose again, then declined between 24 and 72 hours (Lai *et al.*, 1992).

The activity of the enzyme ADH has been observed to increase under hypoxia in the roots of many crop plant species, including rice, which is tolerant of prolonged flooding. Increases were observed in barley (Wignarajah *et al.*, 1976; Good and Crosby, 1989), rice (Wignarajah *et al.*, 1976; Cobb and Kennedy, 1987, Xie and Wu, 1989), wheat (Thomson *et al.*, 1989; Waters *et al.*, 1991), *Pisum sativum* (Jenkin and ap Rees, 1983) and maize (Wignarajah and Greenway, 1976; Andrews *et al.*, 1993; 1994; VanToai *et al.*, 1995). Alcohol dehydrogenase activity is also inducible in soybean seedling roots by flooding (Russell *et al.*, 1990; Newman and VanToai, 1991), despite an earlier report to the contrary (Brzezinski *et al.*, 1986). Alcohol dehydrogenase activity has also been observed to increase in the roots of aquatic plants such as *Echinochloa* sp. (Cobb and Kennedy, 1987), *Ranunculus sceleratus* (Smith and ap Rees, 1979), *Glyceria maxima* (Jenkin and ap Rees, 1983; Smith and ap Rees, 1979), *Zizania palustris* (Muench *et al.*, 1993), *Senecio aquaticus* (Smith and ap Rees, 1979), *Spartina alterniflora* (Mendelssohn and McKee, 1987) and *Schoenoplectus lacustris* Palla (Monk and Braendle, 1982).

Figure 2-4a is a representative western blot of ADH protein in seedling roots during the first four days of treatment. This period was examined to further explore the nature of the changes in ADH activity. The size of the ADH subunits was estimated to be 40 kD, based upon migration in SDS-PAGE, in comparison to polypeptide molecular weight standards. This size is similar to that seen in other plant species, for example,

maize (40 kD) (Schwartz and Endo, 1966), wheat (40 kD) (Mitchell *et al.*, 1989), rice (40 kD) (Xie and Wu, 1989), *Arabidopsis thaliana* (43 to 45 kD) (Dolferus and Jacobs, 1984), and soybean (40 kD) (Brzezinski *et al.*, 1986). Figure 2-4b is a graphic representation of a scanning densitometer analysis of three independent western blots. As expected there was more ADH polypeptide in the nitrogen-treated samples than in both the control roots or the air-treated roots. There is little change in the amount of ADH protein in the hypoxic treatment from day one to day four. The relative abundance of ADH polypeptide in the air-treated samples stayed relatively constant over the treatment period in both the controls and the treated samples. There was no evidence of a biphasic trend in the pattern of ADH polypeptide accumulation, as was seen in the ADH actvity. These results suggest that the amount of ADH protein does not necessarily control the ADH activity levels. Comparison between Figure 2-3 and Figure 2-4b show a decrease in activity at day three without a corresponding decrease in the amount of ADH protein.

This relationship was further examined in Figure 2-5a in the hypoxic and well-aerated treatments, where each sample was assayed for ADH activity and for the amount of ADH protein. Again, a similar trend was observed. From the ADH activity data, there is a drop in the ADH activity in the N_2-treated roots on day three, which was not reflected in the amount of protein on the western blot. A more a rigorous approach to comparing protein and activity would have been to test the ADH activity of all the samples used for the three western blots from Fig. 2-4. Unfortunately, though, this was not possible because of time constraints. This result is different from that of Good and Crosby (1989) who examined the amount of ADH protein in the seedling roots of barley under hypoxia. They observed that the changes in ADH activity were matched by changes in the levels of the polypeptide, indicating that the increase in activity resulted from *de novo* synthesis of the protein. However, they did note that the amounts of ADH polypeptide in developing seedlings was higher than they had expected from the activity measurements, suggesting that an appreciable amount of ADH protein may have been in an inactive form.

Nondenaturing PAGE of ADH isozymes in seedling roots was performed to determine whether the changes in activity were related to changes in the isozyme profile (Figure 2-6). Seedling roots were treated with either N_2 gas or air for up to seven days in the Hydroponic Treatment System. After one day of nitrogen treatment, there was a single isozyme band that was not present in the air treatment. The isozyme band reached a maximum intensity after five days treatment and decreased slightly by day seven. Therefore, no differences in isozyme number were noted in the seedling roots in this treatment system. This pattern of a single ADH band was similar to that seen both *A. thaliana*, (which has only one *Adh* gene) (Dolferus and Jacobs, 1984) and in soybean (which has two *Adh* genes) (Russell *et al.*, 1990; Newman and VanToai, 1991; 1992). In peas, (which have three *Adh* genes) three isozymes were detected in the roots of young seedlings, and these increased in activity three to four-fold with anaerobic induction (Llewellyn *et al.*, 1987).

In contrast, Good and Crosby (1989) observed six bands of ADH activity in N_2-treated seedling roots of barley, as compared to only one under well-aerated conditions. Thus, the hypoxia treatment resulted in the induction of five isozymes not seen under well-aerated conditions. The six isozymes in the hypoxic roots were formed from the random dimerization of the polypeptides encoded by three different *Adh* genes, Adh_1, Adh_2 and Adh_3. In seedling roots of maize (which has two *Adh* genes) subjected to anaerobiosis, there are three isozyme bands (Freeling and Schwartz, 1973). In roots of rice (which has two *Adh* genes), ADH $_{2/2}$ was predominantly expressed (Xie and Wu, 1989) and in wild rice (*Zizania palustris*), a single isoform of ADH was seen in root tissue under hypoxic conditions (Muench *et al.*, 1993).

After seven days treatment in the Hydroponic Treatment System, seedling roots treated with N_2 gas developed adventitious roots from two locations on the seedling hypocotyl (Figure 2-7). By day five, there was evidence of the adventitious root primordia emerging from the hypocotyl. Some of the roots emerged from the base of the hypocotyl, near the neck region, above where the primary root begins and others formed

higher (up to 2 cm) on the hypocotyl. No adventitious roots were observed on the seedlings treated with air or in the control seedlings.

Adventitious roots are defined as those roots that arise in unusual positions or tissues of the plant (Fahn, 1990). The formation of adventitious roots is well correlated with hypoxia and flooding in dicotyledonous plants. The development of adventitious roots has been noted in flooded sunflower plants *(Helianthus annuus)* (Phillips, 1964a; 1964b; Wample and Reid, 1979), mungbean (*Vigna radiata*) (Batten and Mullins, 1978) and tomato (*Lycopersicon esculentum*) (Jackson and Campbell, 1975). Adventitious roots may arise from the stem or hypocotyl, large roots, leaves and callus tissue. Some plants such as willow (*Salix* sp.) have preformed adventitious root initials which are formed from secondary parenchymatous tissue in the leaf or branch gaps (Fahn, 1990).

There is considerable evidence that internal, longitudinally connected, intercellular spaces (lacunae) caused by cell separation (schizogenous aerenchyma) or by breakdown of cells (lysigenous aerenchyma) in the cortex aids root growth and survival in oxygen deficient surroundings (Jackson, 1985). The lacunae facilitate gas diffusion between the root and the aerial portion of the plant, while also decreasing the total O_2 demand. The development of aerenchyma tissue in adventitious roots was correlated with flooding responses in maize (Drew *et al.*, 1979; 1980; 1981; Konings, 1982; Konings and de Wolf, 1984) and rice (Jackson *et al.*, 1985). A large amount of work has been done on the formation of lysigenous aerenchyma (for example: Drew *et al.*, 1979; 1980; 1981; Konings, 1982; Konings and de Wolf, 1984).

Figure 2-8 shows the formation of intercellular air spaces (schizogenous aerenchyma tissue) in adventitious roots of seedlings that have been treated with nitrogen gas for seven days. The adventitious roots from near the neck region (Figure 2-8c) of the hypocotyl have larger lacunae than do the ones from higher on the hypocotyl (Figure 2-8d). Very little is known about the inductive mechanisms of the schizogenous type of

aerenchyma, which also develops extensively in some aquatic species such as *Rumex* (Voesenek *et al.*, 1992).

Conditions such as hypoxia or anoxia limit energy production and simultaneously reduce the respiratory rate and the value of the adenine nucleotide (AdN) ratios (Pradet and Raymond, 1983). As well, the size of the Total Adenylate (TA) pool ([ATP] + [ADP] + [AMP]) may decrease under stress, if the metabolic processes of the plant are adjusted. In N_2-treated samples, the size of the TA pool increased slightly over the first five days of the treatment period (Figure 2-9). This may possibly indicate that the seedling roots have acclimated to the hypoxic stress treatments. In the air-treated and control roots, the TAs are at a maximum after one day treatment and then decrease slightly. The size of the TA pool in N_2-treated samples never fell below 20 pmol/mg protein (the minimum level in the air-treated samples) which may suggest that the seedlings were not severely stressed by the hypoxia treatment.

The relative amounts of the three AdNs are also shown in Figure 2-9. Since ATP makes up the majority of the TAs, the amount of ATP followed the trends seen for the TAs. In the N_2-treated samples, the amount of ATP increased slightly over the first five days of the treatment period. In the air-treated and control roots, the maximum amount of ATP was seen after one day of treatment and then decreased slightly. These result contrast with the findings of Mendelssohn and McKee (1987) who measured the energy status and TAs in roots of the aquatic plant *Spartina alterniflora* after short and long-term hypoxia treatments. They found that after five days hypoxia treatment, the ATP concentration was significantly lower than in the control roots, despite the fact that there were little to no differences in the size of the total adenylate pool. After eight weeks of flooded treatment, the plants had adapted and there were no significant differences in the concentrations of the individual adenylates.

Changes in TAs and ATP appeared to follow a trend similar to ADH activity. There was a large increase in the amount of ADH enzyme activity in the N_2-treated roots

at day four and five (Figure 2-3) corresponding to high levels of ATP and TAs in the five day roots. Similarly, ADH activity peaked after one day in the air-treated and control samples, corresponding to high levels of ATP and TAs. In excised maize adventitious root segments under anaerobic conditions, Drew and co-workers (1985) saw evidence that the induction of the fermentation pathway under conditions of anoxia or hypoxia contributed to the survival of plant material by maintaining the level of adenylates. They observed that anoxia caused a reduction in the ATP concentration to about 5% of that in the aerobic roots. Under anaerobic conditions, sodium fluoride (NaF) treatment (used to inhibit the fermentative pathway) inhibited ATP synthesis, resulting in a corresponding rise in AMP content and depressions in both the ATP/ADP ratio and the AEC ratio. The NaF treatment had no effect upon the nucleotide content of the roots under aerobic conditions.

The Adenylate Energy Charge (AEC) is a ratio of the energy-storing adenylate nucleotides ATP, ADP and AMP (Atkinson, 1969). It is a widely used ratio that is used to estimate the growth and /or metabolic activity of bacterial cultures, plant and animal cells, and even mycoplasmalike-organisms (Salisbury and Ross, 1978). It is based upon evidence that when the AEC ratio is high, enzymes involved in biosynthetic (anabolic) or other ATP-requiring pathways have higher activities and enzymes involved with regeneration of ATP show reduced activity (Ball and Atkinson, 1975). Most actively growing cells have AEC ratio values between 0.8 and 0.95, but lower values have been found in anaerobic or starved cells (Salisbury and Ross, 1978). The AEC ratio of non-stressed, actively metabolizing tissues has been reported to be at least 0.8, and often nearer to 0.85 (Bieleski, 1973; Pradet, 1967) and values around 0.9 were reported in root tips of maize (Raymond *et al.*, 1978; Saglio and Pradet, 1980; Saglio *et al.*, 1980).

The AEC ratio was measured over a seven day treatment period in the seedling roots to determine if the roots were experiencing stress conditions (Figure 2-10). The AEC ratio of the seedling roots remained in the range of 0.80 to 0.95 over the treatment period for all the samples, showing few significant differences (see Anova table in Appendix). The AEC ratio of the N_2-treated roots was slightly lower than that of the

controls at day five, as a result of a small accumulation of AMP (Figure 2-9). The air-treated samples, which had a smaller amount of TAs at day five (but very low AMP levels), had a slightly higher AEC ratio, similar to the control roots. Nonetheless, the AEC did not drop below the level associated with stress at any time during the hypoxia treatment. This may possibly indicate that the seedling roots were able to acclimate to the hypoxic stress, through slowed growth and induction of ADH enzyme activity in the nitrogen-treated samples.

There is a large amount of evidence that there is a quantitative relationship between the energy charge value (AEC ratio) and the level of metabolic activity via fermentative pathways in plants under anoxic or hypoxic stress. For example, in excised maize root, the fermentative capacity can be reduced by depleting the sugar content of the roots by aerobic aging (Saglio *et al.*, 1980). This treatment demonstrated a correlation between AEC and fermentative activity. In cold-hardened winter wheat seedlings, an exposure to a low temperature flooding pretreatment (hypoxia) resulted in an acceleration of glycolysis and alcoholic fermentation and led to higher AEC ratios (Andrews and Pomeroy, 1989). In rhizomes of the aquatic plant *S. lacustris* (bulrush) the AEC ratio initially dropped under anoxia, but subsequently increased to 0.75 (Monk and Braendle, 1982). This acclimation to the anoxic conditions was correlated with an 2.5-fold increase in alcoholic fermentation rates. A similar response was seen under root hypoxia in *Spartina alterniflora* (Mendelssohn and McKee, 1987). Root energy status (AEC ratios) in hypoxically-treated roots were maintained at similar levels (0.85) as those found in aerobically grown roots. They also observed a six-fold increase in ADH activity over the aerated controls. In the present study, the lack of significant changes in AEC ratio made it difficult to determine whether a relationship existed between the ADH activity and the AEC ratio.

In conclusion, it appears as though the seedling roots may be able to acclimate to the hypoxic stress situation. This acclimation response involves both morphological and biochemical changes and may protect the seedlings from experiencing severe stress. This

was indicated by the maintenance of the AEC ratio above 0.8 and the size of the TA pool above 20 pmol/*u*g protein. Firstly, the seedlings reduce their rate of growth. At the same time, there is a biphasic induction of ADH activity. Interestingly, the amount of ADH protein did not appear to correlate with the changes in the ADH activity. There was a single ADH isozyme in the seedling roots, that was induced by hypoxia. Again, quantitative changes in the enzyme did not follow changes in ADH enzyme activity. Finally, the seedlings developed adventitious roots under hypoxia that showed evidence of schizogenous aerenchyma tissue.

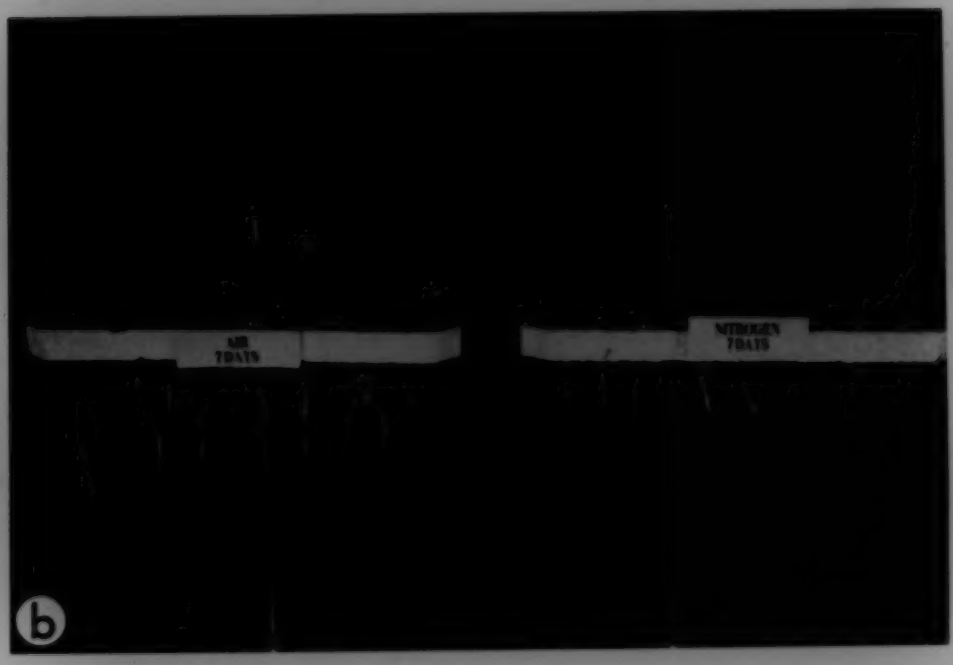
Figure 2-1a. Hydroponic Treatment System.

Greened seedlings (three to four days old) were individually inserted into foam plugs and placed into Styrofoam rafts floating in half-strength Hoagland's solution in rectangular, polyethylene containers (4.0 L, 14 cm by 40 cm). Treatment gas was supplied to each container through a Nalgene tubing line from a gas cylinders, through a small hole, to a 15 cm aerator stone in the nutrient solution. The roots of the seedlings were suspended in the nutrient solution which was sparged continuously with either nitrogen gas or air, to create either hypoxic or well-aerated growth conditions, respectively.

Figure 2-1b. Effects of seven days hypoxia treatment on root growth.

Seedlings are shown after seven days treatment with either nitrogen gas or air. The seedlings in the air treatment air larger and there is substantially more root material than in the nitrogen treatment.

NITROGEN
7DAYS
AIR
7DAYS
a


AIR
7DAYS
NITROGEN
7DAYS
b

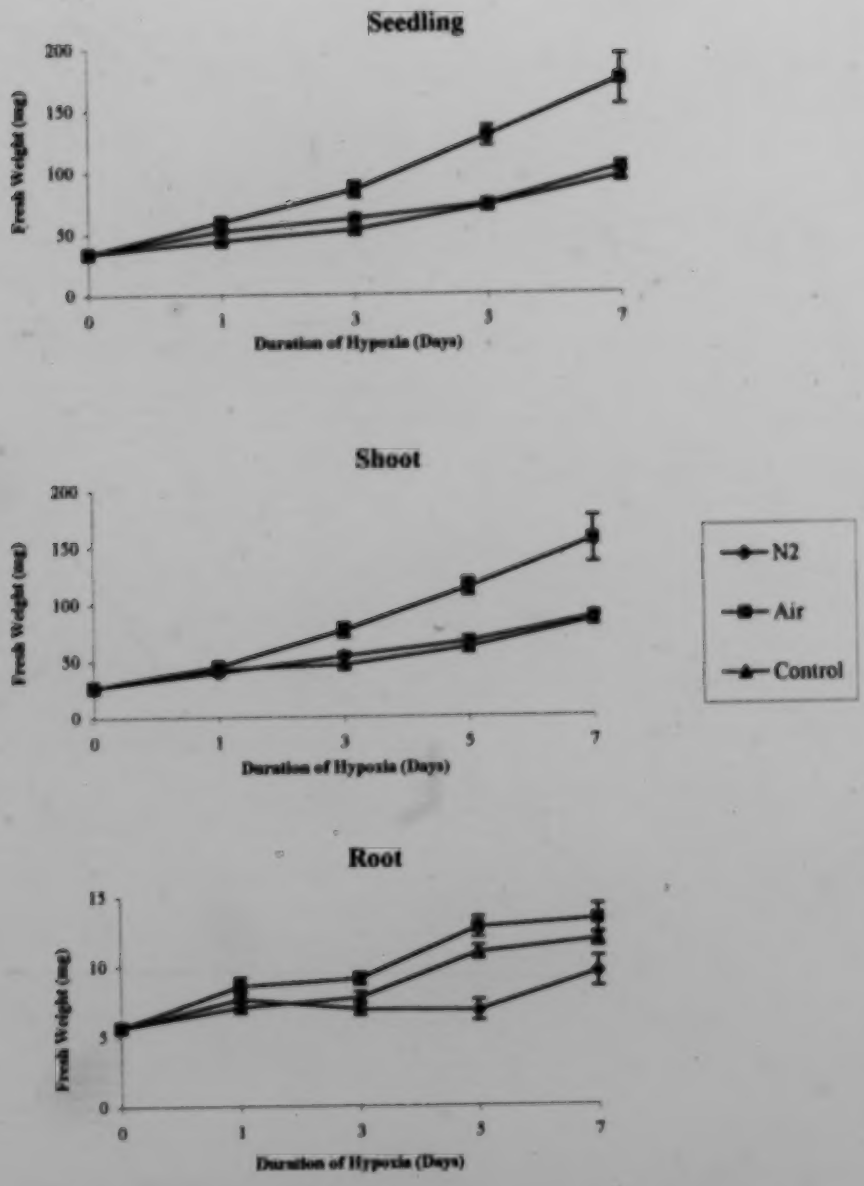


Figure 2-2a. Fresh weight of seedlings, shoots and roots after hypoxia treatment.

Each data point is the mean of three independent experiments (of two samples each), with the standard error of the mean.

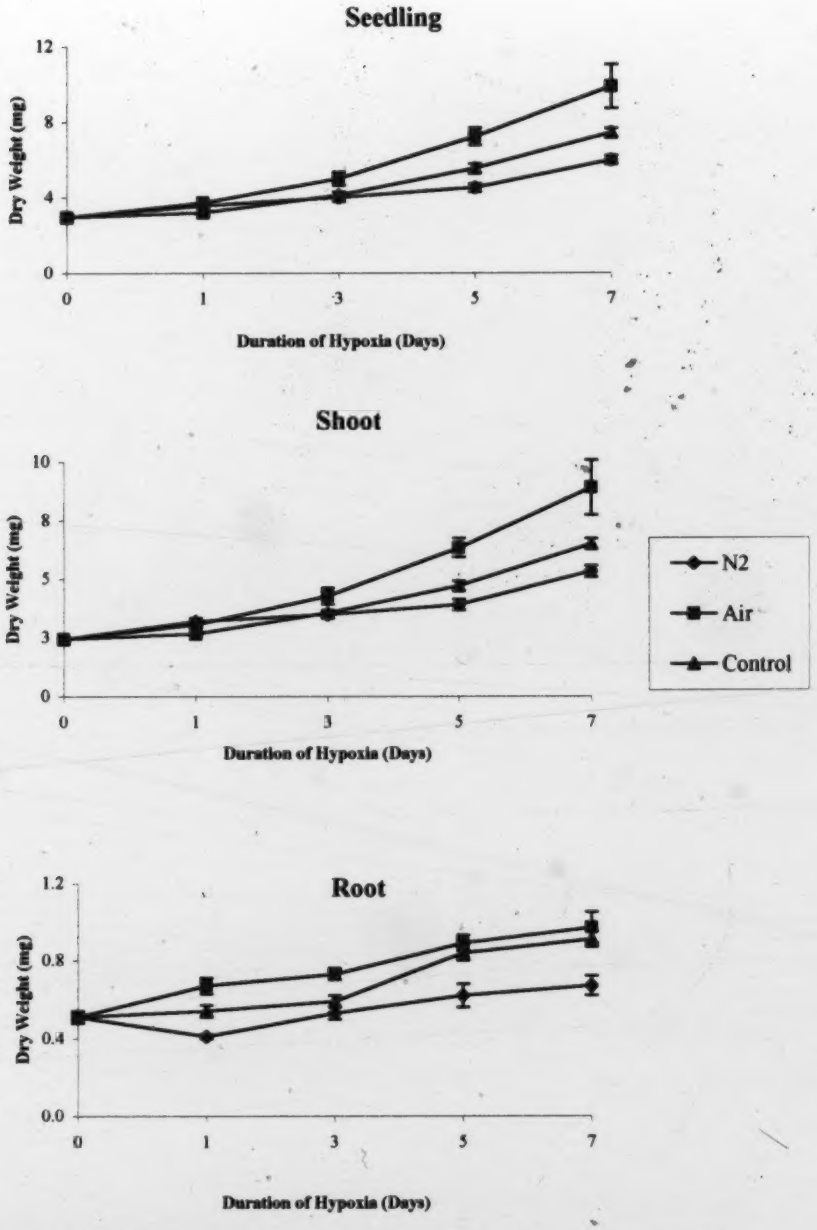


Figure 2-2b. Dry weights of seedlings, shoots and roots after hypoxia treatment.

Each data point is the mean of three independent experiments (of two samples each), with the standard error of the mean.

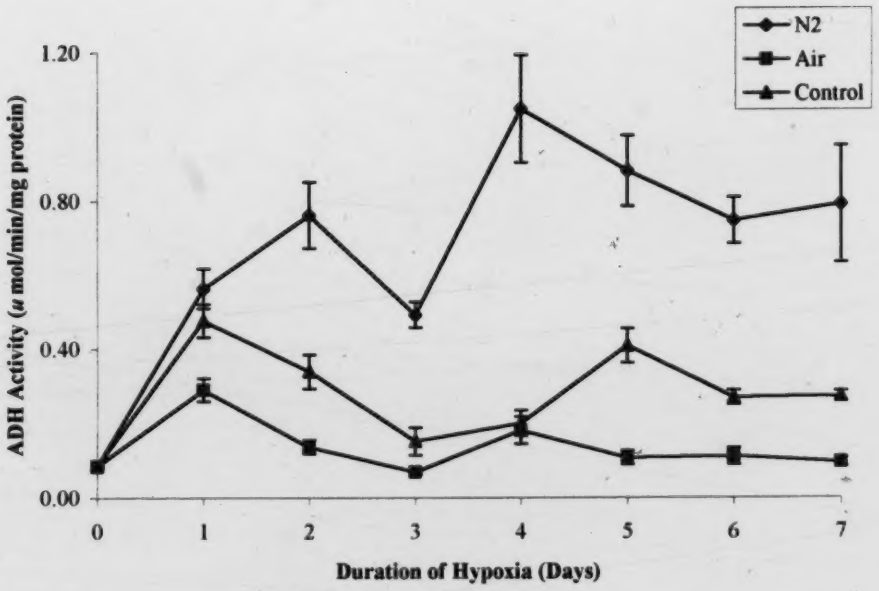


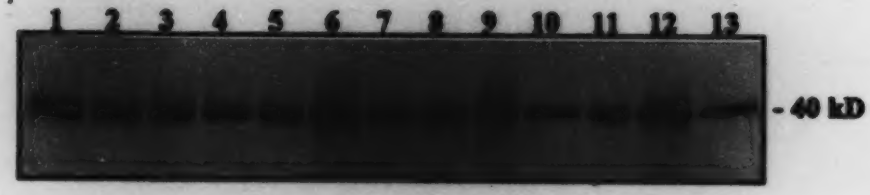
Figure 2-3. Alcohol dehydrogenase activity in seedling roots after hypoxia treatment.

Seedling roots were treated with either nitrogen gas or air in the Hydroponic Treatment System for up to seven days. Control seedlings were grown in petri plates of perlite. Each data point is the mean of three independent experiments (of two samples each), with the standard error of the mean.

Figure 2-4a. Western blot of ADH polypeptide in seedling roots after one to four days hypoxia treatment.

Seedling roots were treated for one to four days in the Hydroponic Treatment System with either nitrogen gas or air. Control seedlings were grown in petri plates of sterile perlite and were wick-watered with half-strength Hoagland's solution. Equal amounts of total soluble protein (30 *u*g) were separated by SDS-PAGE and electroblotted to nitrocellulose membranes.

Lane #	# Days	Treatment
1	0	Control
2	1	Control
3	1	N_2
4	1	Air
5	2	Control
6	2	N_2
7	2	Air
8	3	Control
9	3	N_2
10	3	Air
11	4	Control
12	4	N_2
13	4	Air


1 2 3 4 5 6 7 8 9 10 11 12 13
- 40 kD

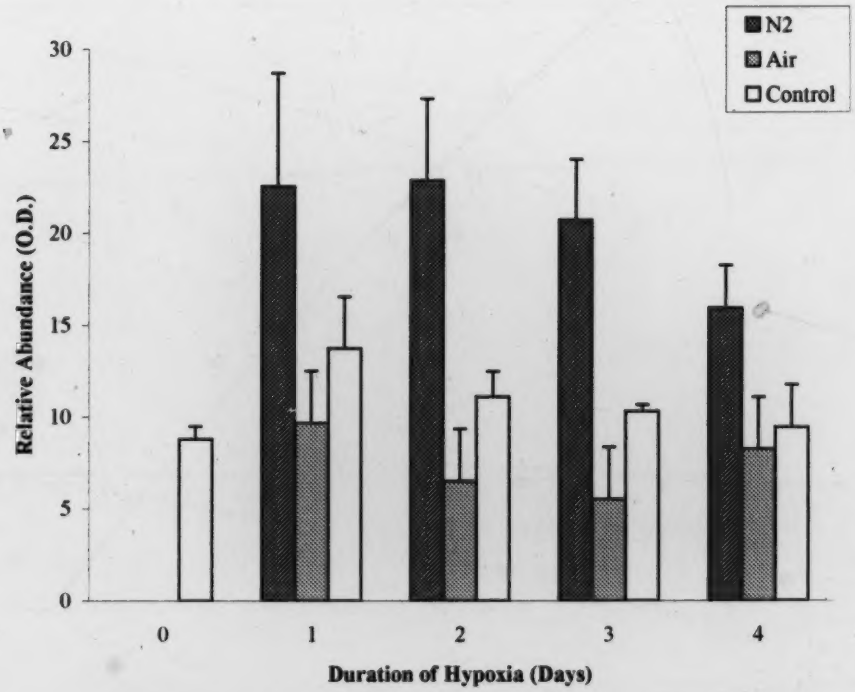


Figure 2-4b. Summary of densitometer scans of 3 western blots of ADH in seeedling roots from day 0 to 4.

This is a graphic representation of the mean Relative Abundance of ADH polypeptide in three western blots, based upon scanning densitometry. All scans were within the linear range of the instrument. The standard error of the means is shown.

Figure 2-5. Western blot of ADH polypeptide in seedling roots after one to three days hypoxia treatment.

Seedling roots were treated for one to three days in the Hydroponic Treatment System with either nitrogen gas or air. Equal amounts (10 ug) of total soluble protein were separated by SDS-PAGE and electroblotted to nitrocellulose membranes.

Lane #	# Days	Treatment	ADH Activity (+/- SE) (μmol/min/mg protein)
1	1	N_2	0.68 (0.04)
2	1	Air	0.37 (0.07)
3	2	N_2	0.73 (0.19)
4	2	Air	0.14 (0.10)
5	3	N_2	0.42 (0.04)
6	3	Air	0.05 (0.02)

Table 2-1. ADH activity in seedling root samples from the western blot.

The ADH activity of the samples was normalized based on the protein content (Bradford, 1976) of the sample. Each data point is the mean of three determinations from each sample, with the standard error.

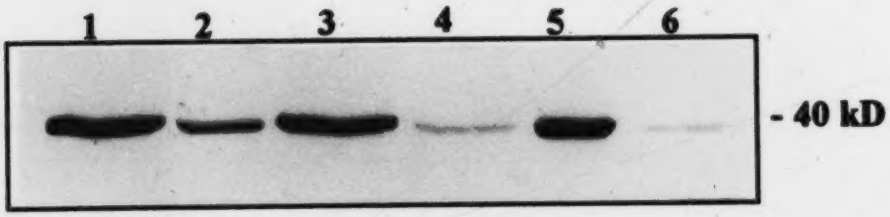

1
2
3
4
5
6
- 40 kD

Figure 2-6. Nondenaturing PAGE of ADH isozymes in seedling roots after hypoxia treatment.

Seedlings were treated in the Hydroponic Treatment System with nitrogen gas for up to seven days. Aliquots containing 25 *u*g of buffer-soluble protein (with 15% glycerol and 0.01% bromophenol blue) were run on a 1.5 mm gel (7.5% acrylamide) under nondenaturing conditions. The gels were run at 4°C, with Laemmli running buffer (Laemmli, 1970) at 75 volts.

Lane #	Treatment
1	Day 0
2	1 Day Air
3	1 Day N_2
4	3 Days Air
5	3 Days N_2
6	5 Days Air
7	5 Days N_2
8	7 Days Air
9	7 Days N_2

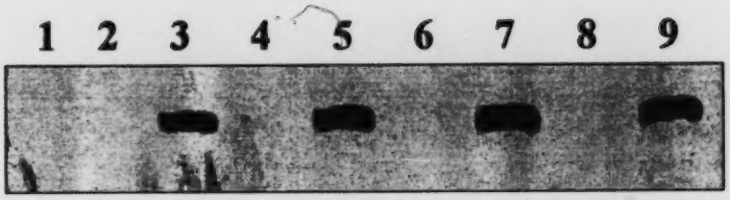

1 2 3 4 5 6 7 8 9

Figure 2-7. Adventitious root formation in hypoxia-treated seedlings after seven days.

Greened seedlings (three to four days old) were treated for 7 days in the Hydroponic Treatment System. The roots of the seedlings were suspended in the nutrient solution which was sparged continuously with either nitrogen gas or air, to create either hypoxic or well-aerated growth conditions, respectively.

Note the development of adventitous roots from two locations on the seedling hypocotyl. Some of the roots arise from the neck region, near the base of the hypocotyl, while others arise from higher up (up to 2 cm from the base) on the hypocotyl.

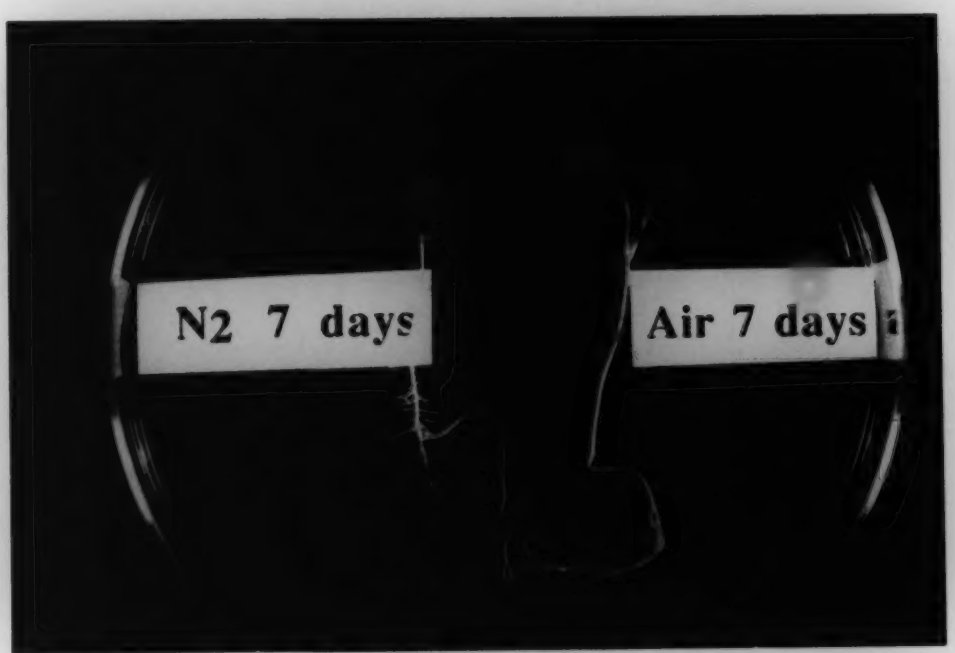

N2 7 days
Air 7 days

Figure 2-8. Formation of aerenchyma tissue in adventitious roots after hypoxia treatment.

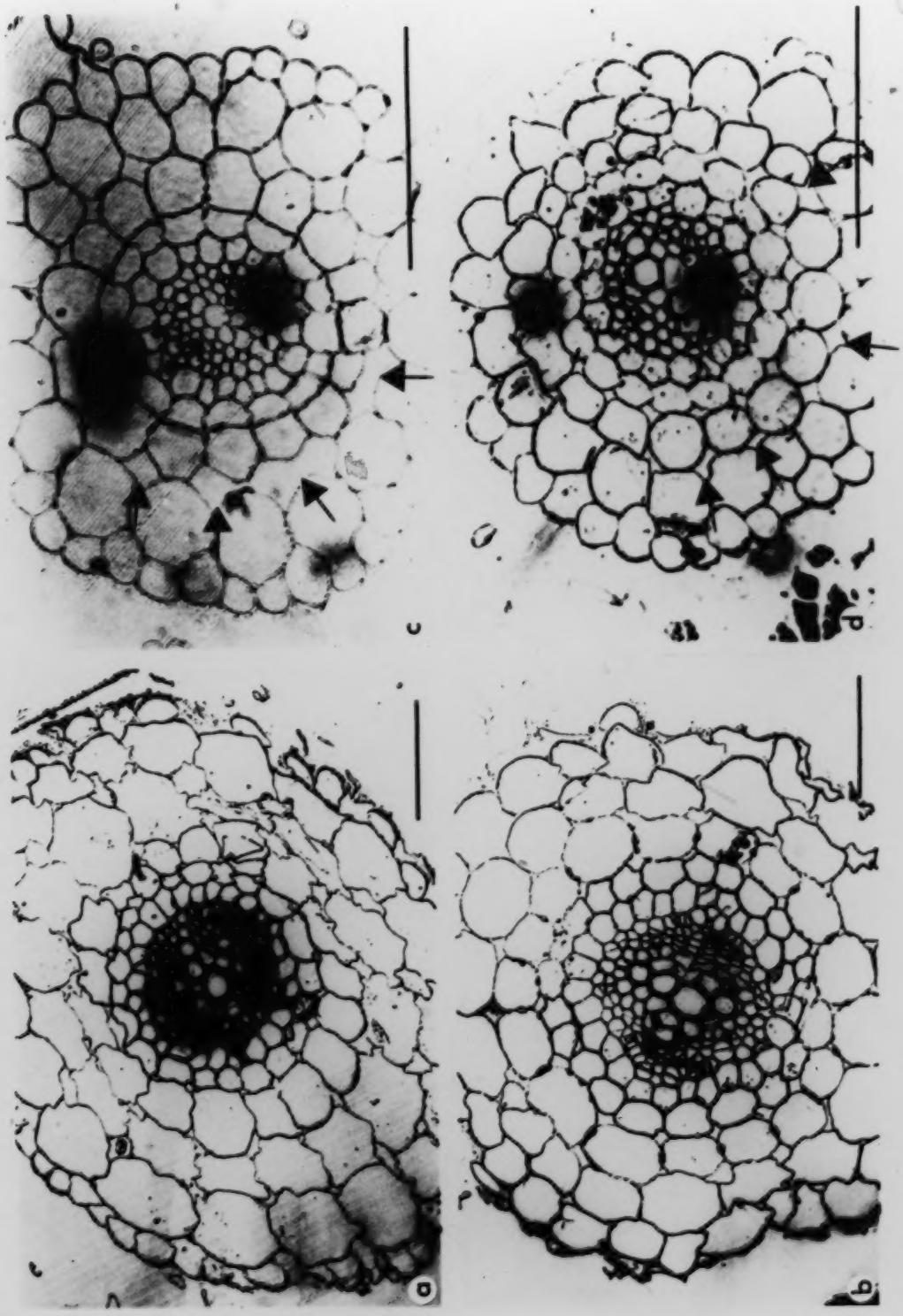
Seedlings were treated in the Hydroponic Treatment System for seven days with either air or nitrogen gas. Primary roots and adventitious roots from both the neck and hypocotyl regions were fixed in 3% glutaraldehyde and embedded in Spurr's Resin (Spurr, 1969). Root sections (1 *u*m each) were stained with 1% toluidine blue in 1% sodium borate and photographed. Lacunae are indicated by arrows. The bars on the pictures are 0.1 mm.

a. Air-treated primary root.

b. Nitrogen-treated primary root.

c. Nitrogen-treated adventitious root from near the neck region.

d. Nitrogen-treated adventitious root from the hypocotyl region.



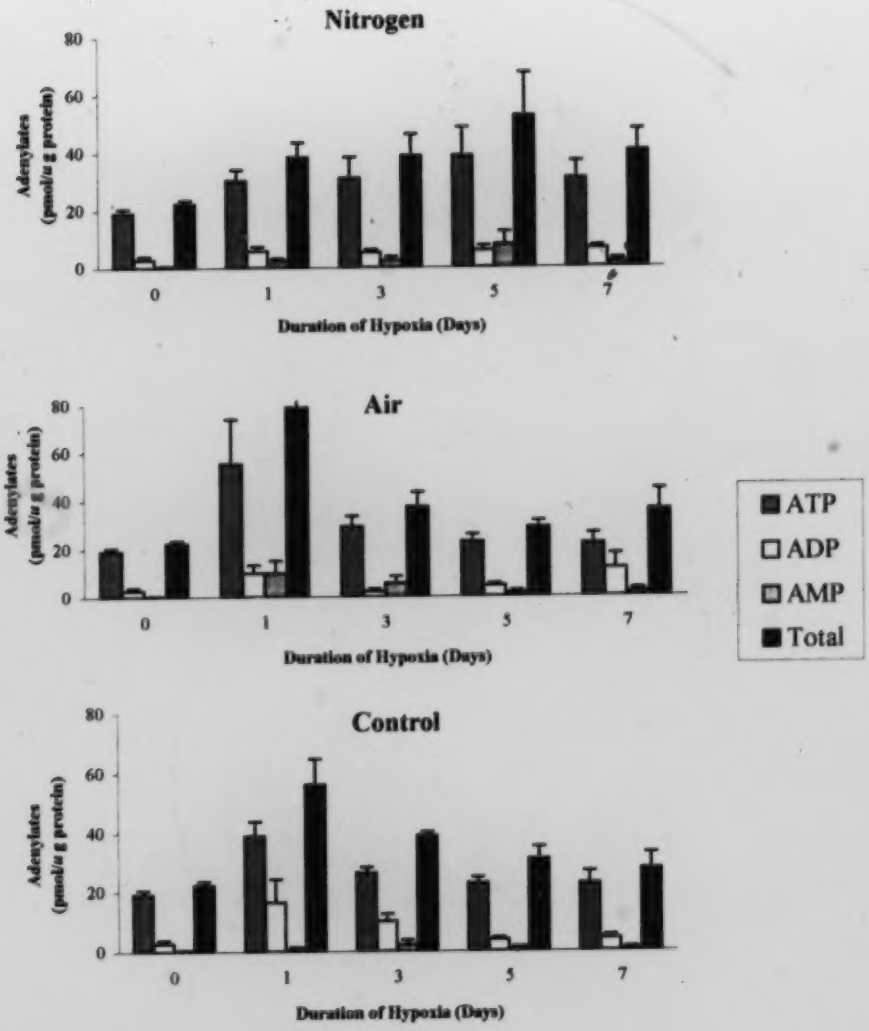


Figure 2-9. Adenylates in seedling roots after hypoxia treatment.

Seedlings were treated in the Hydroponic Treatment System with either nitrogen gas or air for up to seven days. Control seedlings were grown in petri plates on perlite. Each data point was the mean of three independent experiments (of three samples each), with the standard error of the mean.

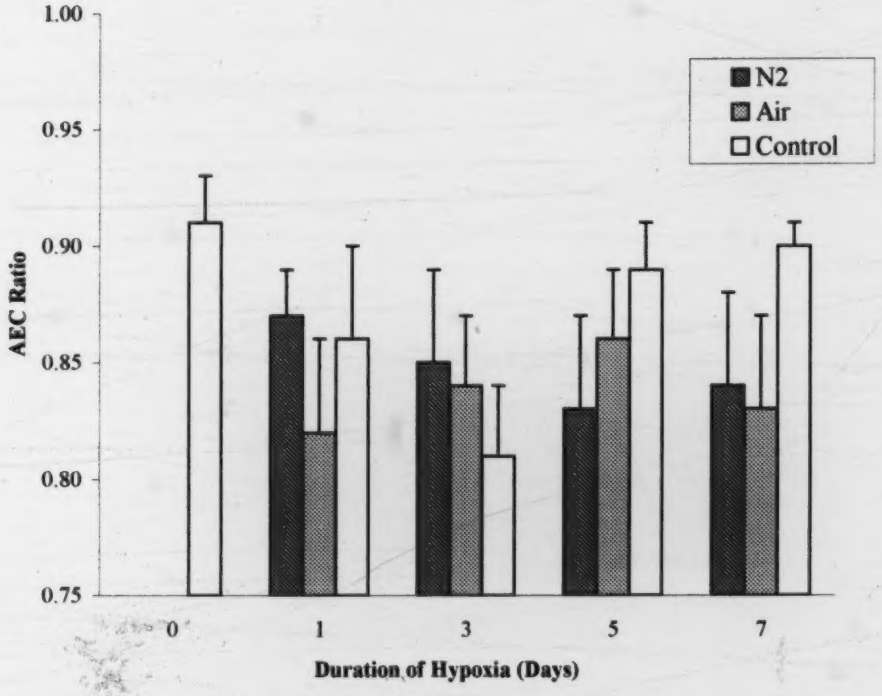


Figure 2-10. Adenylate Energy Charge ratios in seedling roots after hypoxia treatment.

Seedling roots were treated with either nitrogen gas or air in the Hydroponic Treatment System for up to seven days. Control seedlings were grown in perlite in petri plates. Each value is the mean of three independent experiments (of three samples each), with the standard error of the mean.

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Chapter III. Microspore-Derived Haploid Embryos and the Anaerobic Response

Introduction

The work of Guha and Maheshwari (1964; 1966) on anther culture of *Datura innoxia* opened up new possibilities for plant improvement through tissue culture. They showed that anthers or isolated microspores could be cultured to produce haploid plants. The embryos can be regenerated into whole plants that may either undergo spontaneous diploidization, or the chromosome complement can be doubled artificially using colchicine (Coventry *et al.*, 1988). Anther or microspore culture is now performed on a fairly routine basis in a large number of plant species; *Nicotiana*, various members of the Brassicaceae, monocots such as *Hordeum vulgare*, *Oryza sativa*, *Secale cereal*, *Triticum aestivum*, *T. durum*, and *Zea mays*, and the horticultural crops; *Beta vulgaris*, *Capsicum* spp., *Coffea arabica*, *Daucus carota*, *Lycopersicon* spp., *Pisum sativum*, *Solanum* spp. including *S. tuberosum*, and *Vitis vinifera* (for review see; Bajaj, 1990).

Anther or haploid embryo culture, or pollen selection has been used in *Brassica napus* to select for herbicide tolerance (Swanson *et al.*, 1988; Ahmad *et al.*, 1991) and tolerance to fungal pathogens (Ahmad *et al.*, 1991). Selections at the male gametophyte stage are based upon the assumption that many sporophytic genes are also expressed in the gametophyte. Plant regeneration may be less difficult from selections done at the gametophyte stage and the selected traits are more likely to be genetically inherited (Dougall, 1980).

The metabolism of microspore-derived haploid embryos (MDHEs) has received less attention than their utility as material for plant breeding programs. Specific changes in protein synthesis during microspore embryogenesis have been characterized (Cordewener *et al.*, 1994). Wilen and co-workers (1990; 1991) studied the effects of plant growth regulators on the MDHEs. Holbrook and co-workers (1991) characterized the synthesis and accumulation of oilbody proteins in MDHEs of *B. napus*. Taylor and co-workers

(1990) compared storage-protein regulation and storage-lipid accumulation in MDHEs and their zygotic counterparts. The similarities they found in storage-lipid and protein composition, along with the MDHEs' sensitivity to abscisic acid, led them to conclude that MDHEs could potentially be exploited to facilitate studies of biochemistry and gene regulation in oilseeds. Relatively little work has been done on the physiology of the MDHEs and whether they could be used to study plant responses to environmental stresses. The induction of freezing tolerance was studied in MDHEs of *B. napus* (Orr *et al.*, 1990). Recently, Touraev and co-workers (1996) developed a stress-induced microspore embryogenesis system in tobacco for use in biochemical and molecular studies.

In other tissue culture systems, constitutive ADH activity has been observed and this has been attributed to a degree of inherent hypoxia in these systems. Lai and co-workers (1992) observed significant quantities of ADH activity in cell suspension cultures of alfalfa (*Medicago sativa*). The presence of 2,4-dichlorophenoxyacetic acid (2,4-D) in the culture medium may have been a factor in this, as ADH activity has been observed to be induced by 2,4-D in seedling roots and callus cultures (Brzezinski *et al.*, 1986; Freeling, 1973). Callis and co-workers (1987) observed that cultured maize cells were an appropriate system for the study of *Adh*1 expression since ADH is normally expressed there. In tissue cultures of *Arabidopsis thaliana*, very high levels of ADH activity were detected (Dolferus *et al.*, 1985). During callus induction from seeds, on medium containing 2,4-D, ADH specific activity increased during the dedifferentiation process. After a short adaptation period, ADH specific activity in the callus material increased 10-fold. A high level of ADH activity could be maintained by subculturing callus material in suspension cultures (which had lower concentrations of 2,4-D). During exponential growth of the suspension-cultured cells, the ADH specific activity increased again, until the cells stopped dividing and turned brown. The ADH activity in one month old callus was observed to be 10-15 times higher than in plantlets which were anaerobically induced. However, high ADH activity did not preclude further induction. In studies of cultured green algae, *Selenastrum minutum*, Vanlerberghe and co-workers (1990) observed that

ethanol was produced in control cultures (bubbled with air) and was induced about three-fold by anaerobiosis treatment.

The objectives of this study were to determine if MDHEs could be used to study the response of plants to anoxia stress, and to determine if there was sufficient natural variation in the population to permit selection for anoxia tolerance. I was also interested in determining whether the MDHEs would respond more like a seedling or like a zygotic embryo, to gain further insight into the anaerobic response in *Brassica napus*. The relationship between the ability of the MDHEs to survive anoxia treatment and ADH activity was examined. This was studied at the level of ADH enzyme activity, by western blot analysis of ADH protein and by isozyme analysis. Finally, changes in the Adenylate Energy Charge ratio and the size of the Total Adenylate pool were examined.

Materials and Methods

Plant Growth Conditions

(See Chapter II)

Self-Pollination of Plants for Seed Production

(See Chapter II)

Microspore-Derived Haploid Embryo Culture

Microspore-derived haploid embryo cultures were initiated using a modification of the methods of Chuong and Beversdorf (1985) and MacDonald and co-workers (1988). Floral buds from the main raceme and lateral branches were picked immediately before use, at approximately 2-4 mm in length (prior to yellowing). At this time, the petals were 1/2 to 3/4 the length of the anthers. The buds were surface sterilized in 6% sodium hypochlorite (containing two drops of Tween-20 as a surfactant) for 15 minutes, followed

by three rinses (five minutes each) in sterile, deionized water.

Under aseptic conditions, the rinsed buds were gently macerated with a mortar and pestle with 5 mL B_5 media (13% sucrose, without 2,4-D, filter sterilized, pH 6.0) (Gamborg *et al.*, 1968). The homogenate was filtered through a 44 *u*m Nitex nylon monofilament screen cloth (B & S.H. Thompson and Co. Ltd.) and the cellular debris was rinsed with B_5 media. The filtrate, containing the microspores, was collected and the volume was adjusted to 13 mL per 10 buds in sterile, 15 mL Falcon polystyrene tubes (Fisher Scientific Ltd.). The microspore solution was washed three times with 13 mL of B_5 media and centrifuged at 1000, 800, and 500 rpm (145 x *g*, 93 x *g* and 36 x *g*) for five minutes each, in an IEC HN-S bench top centrifuge at room temperature. After the final wash, the microspores were resuspended in 15 mL NN media (pH 6.0, filter sterilized) (Nitsch and Nitsch, 1967), as modified by Lichter (1981) (without Difco potato extract or the hormones 2,4-D, 1-naphthalene acetic acid and 6-benzyl-aminopurine and with 13% sucrose). The resuspended microspores were divided equally between three 60 x 15 mm petri plates. The density in the petri plates was approximately 80,000 microspores per mL NN media (based upon hemacytometer counts). The plates were sealed with Parafilm M laboratory film (American National Can, Greenwich, CT) and incubated for 14 days in the dark at 30°C, after which they were transferred to a rotary shaker (80 rpm) for approximately two to three weeks at 25°C, in darkness.

Microspore-derived Haploid Embryo Treatment in the Anoxia Culture System

Microspore-derived haploid embryos were selected for study at the late torpedo to early cotyledonary stage by filtering the embryos through a coarse Nitex nylon monofilament screen (screen size: 782 *u*m) (B & S.H. Thompson and Co. Ltd.). The selected MDHEs were subcultured to 10 mL fresh NN media in sterilized 25 mL sidearm Erlenmeyer flasks fitted with rubber bungs and fermentation locks (Figure 3-1a and 3-1b). The media was replaced every five days. Fermentation locks prevented contamination while allowing the treatment gas to pass through the cultures. The density of MDHEs in the flasks was empirically determined and was approximately the same in all treatments.

The flasks were covered with aluminum foil to exclude light. A constant flow of either nitrogen gas (extra dry, minimum purity: 99.95% N_2, Linde, Union Carbide) or air (extra dry, Linde, Union Carbide) was passed through a cotton plug and bubbled continuously through the culture medium to create either anoxic or well-aerated conditions (air-treated), respectively. The gas flow rates were approximately 100-105 mL/min per flask (as measured using a Fischer and Porter Flow Meter). The gas flows were humidified by bubbling through sterile, deionized water, before entering the flasks. The concentration of dissolved oxygen, as measured in the NN media in the flasks at 22.2°C using a YSI Oxygen Probe and Meter, model 54A, was 1.8 mg/L (N_2 treatment) (4.6% O_2) and 6.7 mg/L (air treatment) (17 % O_2). Control embryos were grown in 60 x 15 mm petri plates, (in darkness, 25°C), on a rotary shaker (80 rpm) after being subcultured into 10 mL fresh NN media.

Survival Assessment of the MDHEs

After treatment in the Anoxia Culture System for the specified period of time, the MDHEs were removed from the flasks and plated on Regeneration Media (B_5 media with 2% sucrose, 0.8% agar) (Coventry *et al.*, 1988). The petri plates were placed onto a light bench (100 *u*mol m^{-2} s^{-1}, 16 hour photoperiod, 25°C) and after three days, the survival of the embryos was evaluated according how they responded to the anoxia stress. A preliminary study had determined that only Group I embryos would survive past 14 days. The survival assessment was necessary because of the variable nature of the MDHEs response. Group I embryos were therfore assessed as survivors. For each time point (except for day 11), three independent experiments were performed, consisting of one sample each (n=3). Each sample consisted of one flask with approximately 100 to 200 haploid embryos. The 11 day treatment was only replicated twice (pseudoreplication) because of the inherent problems of maintaining sterility over such a long treatment. As a result, the standard error of this treatment date was not included, and inferential statisitics were not performed on this data.

Response Groups:

I- Cotyledons greened, primary root growing, many lateral roots.

II- Cotyledons white, hypocotyl developing callus and greening, primary root growing, many lateral roots.

III- Cotyledons white, no callus growth or greening in hypocotyl region, primary root growing, no lateral roots.

IV- No growth or greening, embryos dark-colored.

ADH Enzyme Assays

Alcohol dehydrogenase (ADH) enzyme activity was determined as in Chapter II. Each data point was the mean of three independent experiments, consisting of two samples of haploid embryos each (n=3). The standard error of the mean was calculated. An Analysis of Variance (Anova) was performed to determine if the variation between the treatments was significant (see Appendix).

Western Blots

Microspore-derived haploid embryos were removed from the Anoxia Culture System, and immediately ground in ADH assay grind buffer (see Chapter II). The ground samples were microcentrifuged at 4°C for five minutes at 13,000 rpm (~ 6000 x g). The soluble protein content was determined in an aliquot of the supernatant using the method of Bradford (1976). An aliquot of the supernatant was diluted 1:3 in SDS sample buffer (62 mM Tris-HCl, pH 6.8, 10% glycerol (v/v), 2% SDS (w/v), 5% 2-β-mercaptoethanol with bromophenol blue as a tracking dye). The diluted aliquots were boiled for five minutes and cooled on ice. An equal amount of total buffer-soluble protein (5 *u*g) was separated in Laemmli running buffer (Laemmli, 1970) on a 1.5 mm thick, 12% SDS polyacrylamide gel with a 4% stacking gel (see Chapter II). The gel was polymerized with 0.08% TEMED and 0.05% APS and run at 180 volts (constant voltage) using a Bio-Rad Minigel apparatus. Western blotting was performed as described in Chapter II. Three independent replications of western blots were analyzed using a Bio-Rad Imaging

Densitometer, Model GS-670 and Bio-Rad Molecular Analyst software, version 2.1. All densitometer scans were within the linear range of the instrument.

Nondenaturing PAGE Gel of ADH isozymes

Non-denaturing PAGE of isozymes of ADH was performed according to Hanson and Jacobsen (1984). All samples used were *B. napus*, R8311, except for developing seeds of the cultivar 'Westar' and the barley (*H. vulgare* 'Leduc') samples. Seeds were germinated on moist perlite for 24 hours. Microspore-derived haploid embryos were treated in the Anoxia Culture System for three days or grown in petri plates (control). Greened seedlings (three to four days old) were treated in the Hydroponic Treatment System for four days with either N_2 gas or air. Samples were loaded on a fresh weight basis; 'R8311' seedling roots (50 mg), barley seedling roots (25 mg), and seed and MDHE samples (10 mg). Samples were ground in 500 *u*L sample buffer (150 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol) in 1.5 mL microcentrifuge tubes using a Pellet Pestle (Kontes). The crude homogenates were microcentrifuged for five minutes at 4°C at 13,000 rpm (~ 6000 x g). Aliquots (with 15% glycerol and 0.01% bromophenol blue) were run on a 1.5 mm, 7.5% non-denaturing polyacrylamide gel (7.4% acrylamide- 0.1% bis, with 0.38 M Tris-HCl (pH 8.5)) on a Bio-Rad large gel apparatus. The gel was polymerized with 0.08% TEMED and 0.05% APS. The stacking gel contained 4.0% acrylamide-0.63% bis, 75 mM Tris-HCl (pH 8.5), 20% sucrose, polymerized with 0.06% TEMED and 0.05% APS. The gels were run at 4°C, with Laemmli running buffer at 10 mA/gel until the dye front entered the running gel, then at 20 mA/gel for the remainder of the run. The gels were stained as in Chapter II.

Determination of Adenylates

The Total Adenylates and Adenylate Energy Charge were determined as in Chapter II. For each data point three independent experiments (of three samples of haploid embryos) were performed (n = 3). The standard error of the mean was calculated. An Analysis of Variance (Anova) was performed to determine if the variation between the

treatments was significant (see Appendix). This analysis was performed over a seven day period, since the survival rate at nine days was less than 25%.

Results and Discussion

The objectives of this study were to characterize the response of MDHEs to anoxia stress and determine if they would be suitable for selection purposes. I was also interested in determining whether the MDHEs would respond more like a seedling or germinating seed. I was interested in examining the relationship between MDHE survival under anoxia treatment and ADH activity.

The cultures of MDHEs were treated with nitrogen gas in the Anoxia Culture System, as shown in Figures 3-1a and 3-1b. To assess the ability of the MDHEs to survive anoxia, they were removed from the Anoxia Culture System and plated on Regeneration Media. A representative sample is shown in Figure 3-2 after eight days treatment with anoxia. Note the differences between the control and the N_2-treated MDHEs. After three days on Regeneration Media (Figure 3-2a), the control MDHEs had greened and there was evidence of primary and lateral root growth, while the N_2-treated ones showed only a small amount of primary root growth, with no greening of the hypocotyl or cotyledons. These were classed in Response Group III. After 14 days on Regeneration Media (Figure 3-2b), it is evident that the control MDHEs are growing and the anoxia-treated ones are not. Although after eight days of anoxia treatment there was about 25% survival, this is not illustrated in this photo which was chosen to illustrate the extremes of the survival classes.

Survival of the MDHEs was assessed based upon the survival classes observed during the regeneration procedure. Only those MDHEs that fell into Survival Class I (cotyledons greened, primary root growing, many lateral roots growing) were counted as survivors. This method of survival assessment was also used by Sacristan (1982), who based the survival assessment of cell cultures and somatic embryo cultures on subsequent growth after at least two passages on medium containing toxin of *Phoma lingam*. In

another study, Ahmad and co-workers (1991) assessed the survival of young haploid embryoids (*sic*) after being subjected to two cycles on selective media based upon their continued growth and regeneration of plants.

A survival curve was generated (Figure 3-3) over a period of 11 days of anoxia treatment in the Anoxia Culture System. Most (71.2% to 96.6%) of the MDHEs were not viable after nine days in anoxia treatment, compared to 12.2% to 40% of the controls. It was observed that there was a high degree of variability in the survival of the MDHEs after treatment in anoxia, as well in the controls. The presence of this variation in the MDHE populations should make it possible to select from the population for tolerance to anoxia. The variable nature of microspore cultures and haploid embryos have also been observed in other studies. There was up to 40% variation observed in the survival of microspores of *B. napus* after irradiation treatment with ultraviolet light (Ahmad *et al.*, 1991).

The level of ADH enzyme activity was measured daily over an eight day treatment period in anoxia-treated and control MDHEs (Figure 3-4). There was a high, constitutive level of ADH that did not change significantly as a result of the N_2 treatment (see Anova in Appendix). This is different than what was observed in cell cultures of alfalfa (*M. sativa*) (Lai *et al.*, 1992) and in the green algae, *S. minutum* (Vanlerberghe *et al.*, 1990). In both these *in vitro* systems, anaerobiosis treatment resulted in a significant induction of ADH activity and/or ethanol accumulation. The result in the MDHE system also contrasts with the response seen in the seedling roots (Chapter II), where the ADH activity increased sharply with hypoxia treatment. Interestingly, the level of ADH activity observed in the MDHEs was not significantly different to that measured in the seedling roots under hypoxia.

High ADH activity has been shown to confer survival in a number of systems (Johnson *et al.*, 1989; Hole *et al.*,1992; Johnson *et al.*,1994), as evidenced by high AEC ratios and TAs. As such, the haploid embryos should be able to survive up to about eight

days of anoxia. Our results (Figure 3-3) show that this is the case. Survival levels of the N_2-treated MDHEs are not significantly lower than those of the controls until day nine. After this point the survival of the anoxia-treated MDHEs drops to approximately 20%.

Figure 3-5a is a representative western blot showing the relative amount of ADH polypeptide present in the MDHEs under anaerobic or control conditions during the first five days of treatment. The size of the ADH subunits was estimated to be 40 kD, based upon migration in SDS-PAGE, in comparison to polypeptide molecular weight standards. This size is similar to that seen in other plant species, for example, maize (40 kD) (Schwartz and Endo, 1966), wheat (40 kD) (Mitchell *et al.*, 1989), rice (40 kD) (Xie and Wu, 1989), *A. thaliana* (43 to 45 kD) (Dolferus and Jacobs, 1984), and soybean (40 kD) (Brzezinski *et al.*, 1986). Figure 3-5b shows a graphical representation of the mean of three independent western blots, based upon scanning densitometry. There was little change in the amount of ADH over time or among treatments. However, there was a decrease in the quantity of ADH over time in the control samples. In our previous work, (Chapter II), it was observed that ADH activity was independent of the quantity of ADH protein. These results, therefore, further support the observation that quantitative measurement of ADH does not reflect ADH activity. This again raises the question as to how the induction of ADH activity may be regulated.

Isozyme patterns are often unique to specific tissues and developmental phases of the plant. For example, in mature dry seeds of rice, $ADH_{1/1}$ was the predominant isozyme expressed, but after germination in the air, three ADH activity bands appeared (Ricard *et al.*, 1986). In roots of rice seedlings, $ADH_{2/2}$ was predominantly expressed, while $ADH_{1/1}$ was expressed in the leaves, sheaths, and pollen (Xie and Wu, 1989). Conversely, in *A. thaliana*, zymograms of different plant organs; seeds, cotyledons, roots, and callus showed a single isozyme, staining most intensely in the callus material (Dolferus and Jacobs, 1984).

These results suggest that by comparing zymograms of MDHEs, seedling roots and devoloping and germinating seeds, I might be able to gain more insight into the nature of the anaerobic response in MDHEs, and in *B. napus*, in general. Examination of ADH isozymes in MDHEs, seedling roots and seeds (Figure 3-6) indicated that there may be as many as four different isozymes expressed among the different tissues. Developing seeds appear to share a common band with MDHEs, which is not seen in the other tissues. As well, the developing seeds (at 23 and 28 DPA) have a second, unique band. The dry seed and germinated seeds (after 24 hours) also share another unique band, while the MDHEs and seedling roots share yet another band. Thus, the MDHEs seem to share some of the characteristics of both the developing seeds and seedlings.

On the basis of previous results (Chapter II), it appeared that approximately 20 pmol/*u*g protein Total Adenylates (TAs) are characteristic of a non-stressed condition in the seedling roots. The TA pool in the MDHEs was significantly depleted under anaerobic conditions by day five, as compared to the other samples (Figure 3-7). The maximum amount of TAs in the N_2-treated MDHEs occurred at day one (~40 pmol/*u*g protein) and decreased steadily, dropping below 20 pmol/*u*g protein on day five. This was associated with a large drop in survival (Figure 3-3) from day seven (~75%) to day nine (~15%). In the air-treated samples, the level of TAs remained at approximately 20 pmol/*u*g protein or higher and the survival rates were maintained at approximately 100%.

Figure 3-8 shows the changes in the Adenylate Energy Charge ratio over the seven day treatment period after treatment with N_2 gas and air, in comparison to control samples. Essentially, there was little significant change in the AEC ratio. This correlates with the lack of change in ADH activity, and, as such, could support the hypothesis that ADH activity is involved in the maintenance of the AEC ratio. However, AEC ratios were lower than would have been predicted. In the seedling root system, ADH activity in the range of approximately 0.8 to 1.0 *u*mol/min/mg protein was sufficient to maintain the AEC ratio above 0.8. In the MDHEs, however, the same amount of ADH activity resulted in an AEC ratio of generally less than 0.8, which other workers have identified as

the threshold for stress or survival (Raymond and Pradet, 1980; Saglio *et al.*, 1980). At the beginning of the experiment the AEC is rather low, but this may be explained by the relatively high proportion of AMP (Figure 3-7). There was a decrease in the AEC ratio in the N_2-treated haploids from 0.80 at day one to 0.65 at day seven. There was no significant difference in the AEC ratios between the air-treated and the control embryos, over the same time period.

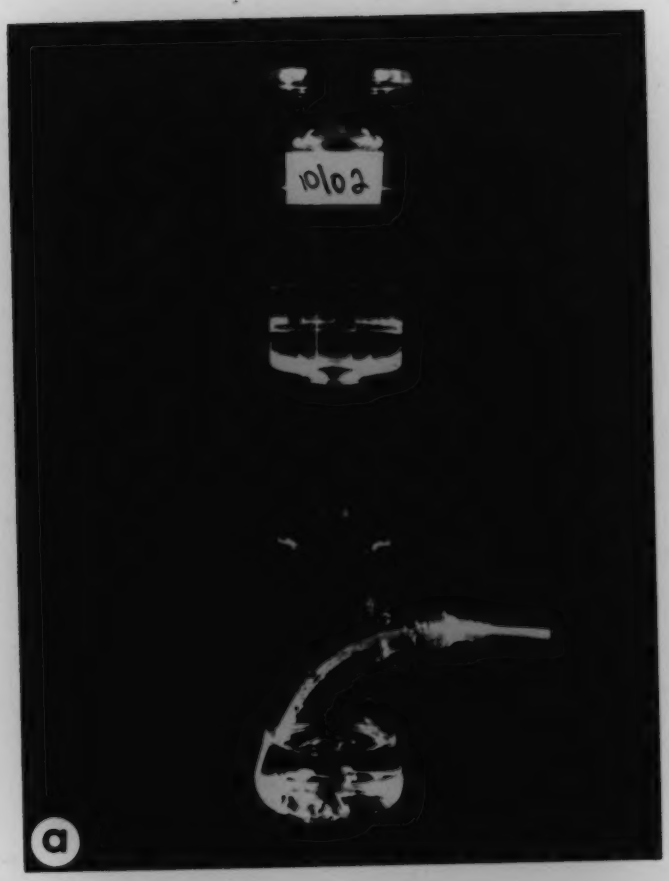
In summary, further insights into the flooding response in *Brassica napus* were gained by the study of MDHEs under anoxia. In this system, (unlike other *in vitro* systems) ADH activity is constitutively high and was not further induced under anoxia. Despite this, the TA levels were not maintained above a threshold level. In addition, the high ADH activity was not sufficient to maintain survival of the MDHEs past nine days. Comparison between ADH activity and the amount of ADH again indicates that the relationship between these two parameters is not as clear as expected. Based upon the zymograms, the MDHEs appear to be intermediate between the seedling roots and the seeds, sharing an isozyme with each. The zymogram also gives further insight into the genetics of the system (allelic differences aside), indicating that it may not be a single gene, but is probably a two or three gene system. Finally, the wide range of survival noted in the present study indicates that there may be potential to use MDHEs as a system to select for anoxia tolerance in *Brassica napus*.

Figure 3-1a. Anoxia Culture System - Single Flask.

Microspore-derived haploid embryos were treated in 10 mL NN media in 25 mL sidearm flasks fitted with rubber bungs and fermentation locks. A constant flow of humidified nitrogen gas or air was passed through a cotton plug and bubbled continuously through the culture medium to create either anoxic or well-aerated conditions, respectively. The aluminum foil was removed from this flask to show the line that supplied the gas flow from the sidearm port.

Figure 3-1b. Anoxia Culture System - Multiple Flasks.

A representative picture of four flasks set up in sequence, showing the gas lines from the tanks. The flasks were covered with aluminum foil to exclude light. Clamps on the lines were used to equalize the gas flow between the flasks.


1012
a

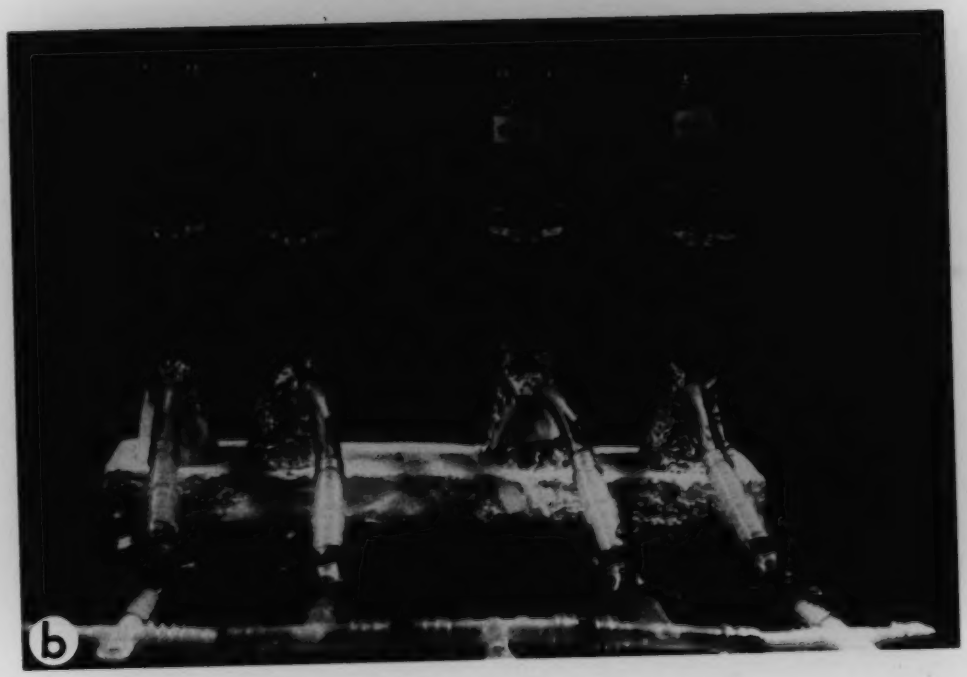
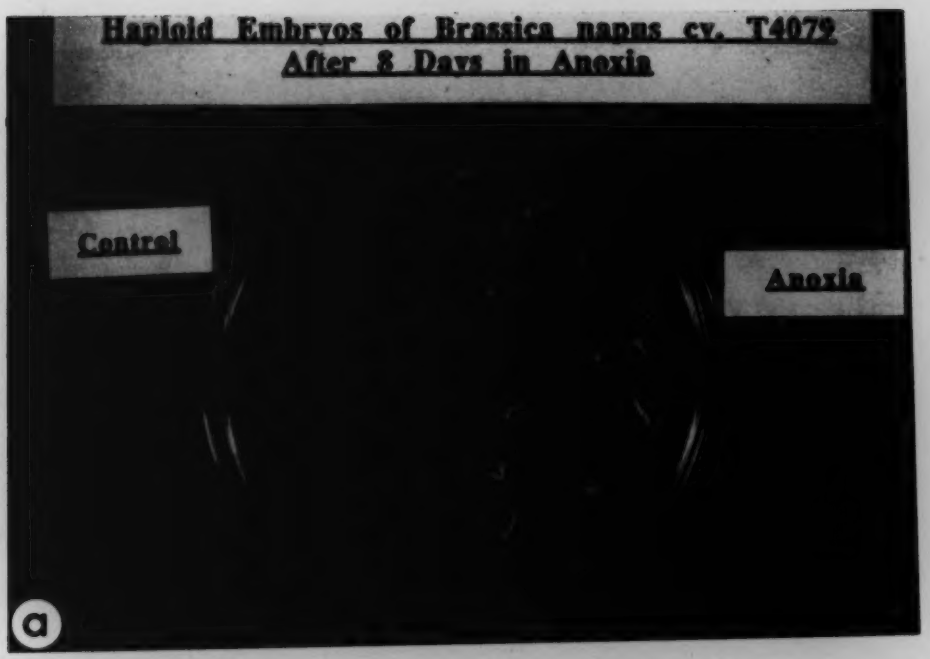
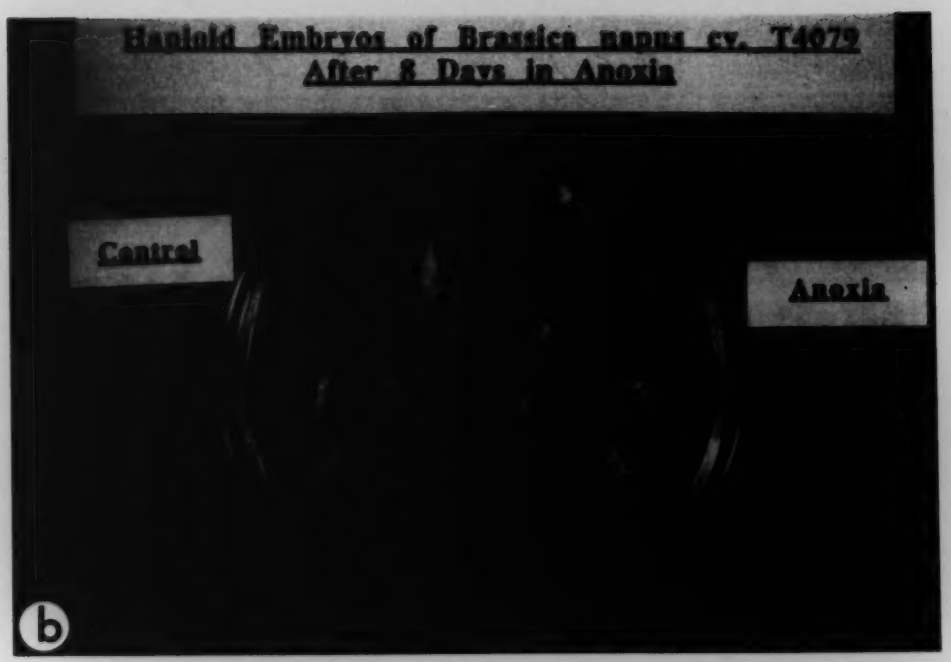

b

Figure 3-2a. Microspore-derived haploid embryos after eight days treatment in anoxia and three days on Regeneration Media.

Microspore-derived haploid embryos were treated for eight days with nitrogen gas (anoxia) in the Anoxia Culture System. Control MDHEs were grown in petri plates and shaken at 80 rpm. After treatment, the MDHEs were plated on Regeneration Media and placed on a light bench and after three days the survival was assessed.

Figure 3-2b. Microspore-derived haploid embryos after eight days treatment in anoxia and 14 days on Regeneration Media.


Haploid Embryos of Brassica napus cv. T4079
After 8 Days in Anoxia
Control
Anoxia
a


Haploid Embryos of Brassica napus cv. T4079
After 8 Days in Anoxia
Control
Anoxia
b

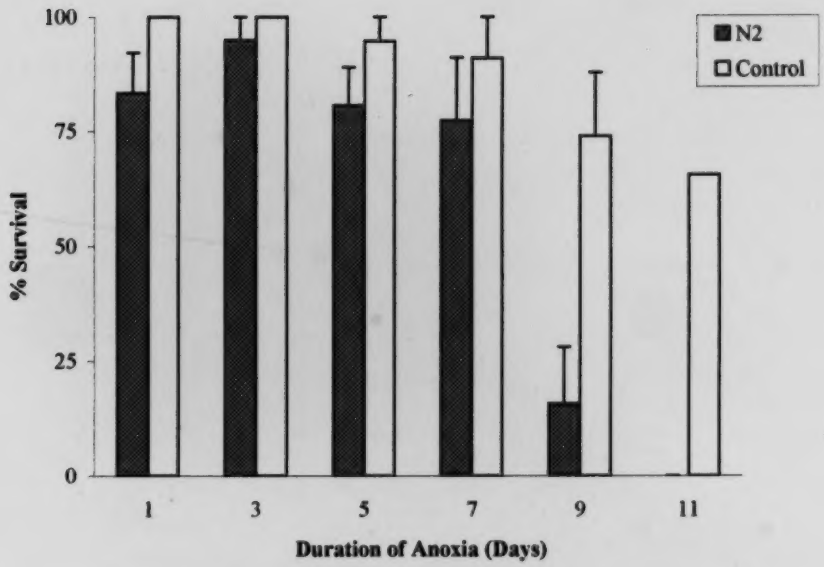


Figure 3-3. Survival of MDHEs after treatment in the Anoxia Culture System.

Microspore-derived haploid embryos were treated for up to 11 days and their survival was assessed on the basis of greening and subsequent growth on Regeneration Media. Control embryos were grown in petri plates and shaken at 80 rpm. For each time point (except for day 11), three independent experiments were performed, consisting of one sample each (n=3). The means of the samples are shown, with the standard error. The 11 day treatment was only replicated twice because of the inherent problems of maintaining sterility over such a long treatment. As a result, the standard error of the day 11 samples was not included.

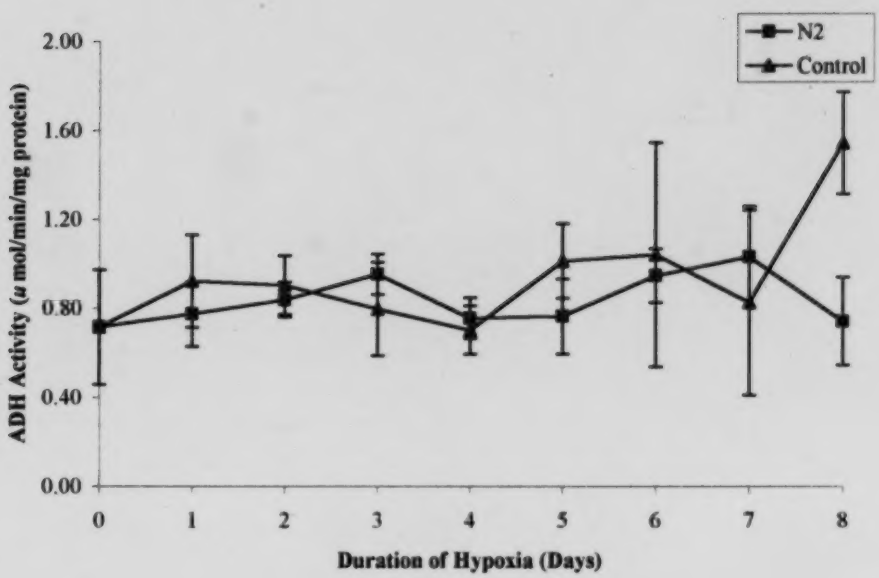


Figure 3-4. Alcohol dehydrogenase activity in MDHEs after treatment in the Anoxia Culture System.

Microspore-derived haploid embryos were treated with nitrogen gas or air in the Anoxia Culture System for up to eight days. Control MDHEs were grown in petri plates and shaken at 80 rpm. Each data point is the mean of three independent experiments (of two samples each) with the standard error of the mean.

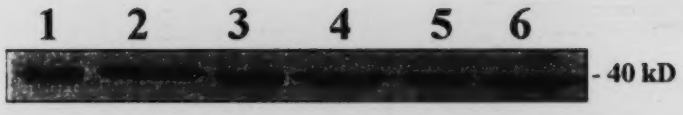
Figure 3-5a. Western blot of ADH polypeptide in MDHEs after treatment in the Anoxia Culture System.

Microspore-derived haploid embryos were treated for 1 to 5 days in the Anoxia Culture System with nitrogen gas (anoxic). Control embryos were grown in petri plates in darkness at 25°C and shaken at 80 rpm. Equal amounts of total soluble protein (5 *u*g) were separated by SDS-PAGE and electroblotted to nitrocellulose membrane.

Lane #	# Days	Treatment	ADH Activity (+/-SE) (*u*mol/min/mg protein)
1	1	N_2	0.83 (0.04)
2	1	Control	0.65 (0.10)
3	3	N_2	0.99 (0.07)
4	3	Control	0.47 (0.04)
5	5	N_2	1.00 (0.09)
6	5	Control	0.53 (0.09)

Table 3-1. Alcohol dehydrogenase activity in MDHE samples used for western blot.

The ADH activity of the samples was normalized based on the protein content (Bradford, 1976) of the sample. Each data point is the mean of four determinations from each sample, with the standard error.


1 2 3 4 5 6
- 40 kD

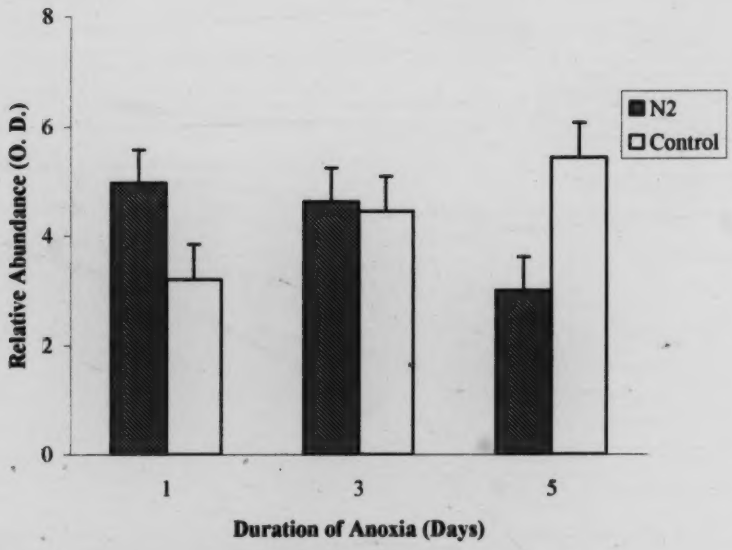
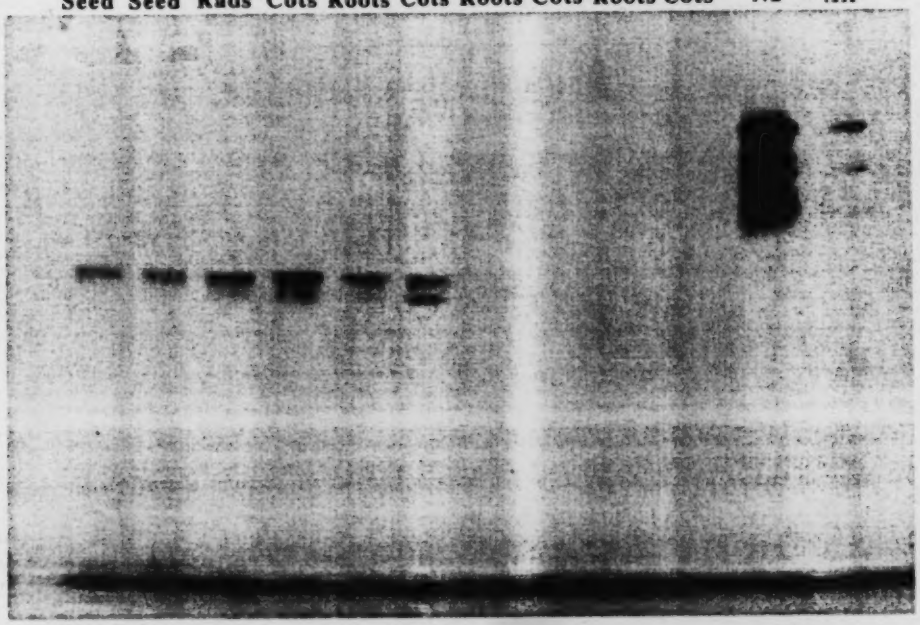


Figure 3-5b. Relative abundance of ADH polypeptide in MDHEs samples.

This is a graphic representation of the mean Relative Abundance of ADH polypeptide in three independent western blots, based upon scanning densitometry. All scans were within the linear range of the instrument. The standard error of the means is shown.

Figure 3-6. Nondenaturing PAGE of ADH isozymes comparing seeds, seedling roots and MDHEs.

Samples were loaded on a fresh weight basis; 'R8311' seedling roots (50 mg), barley seedling roots (25 mg), seed and MDHE samples (10 mg) and were separated on a 1.5 mm, 7.5% non-denaturing polyacrylamide gel with a 4.0% stacking gel. Seeds were germinated on moist perlite for 24 hours. Microspore-derived haploid embryos were treated in the Anoxia Culture System with nitrogen gas or air for three days. Control (Ctrl) MDHEs were grown in petri plates, and shaken. Greened seedlings (three to four days old) were treated in the Hydroponic Treatment System for four days with either nitrogen gas or air. All samples were *B. napus*, 'R8311', except for developing seeds of the cultivar 'Westar' and the seeds and seedling roots of *H. vulgare* 'Leduc' which were included for comparison.


Dry Seed
1 Day
Seed
Rads
Cots
2 Days
Roots
Cots
3 Days
Roots
Cots
4 Days
Roots
Cots
Barley
Roots
N2
Air

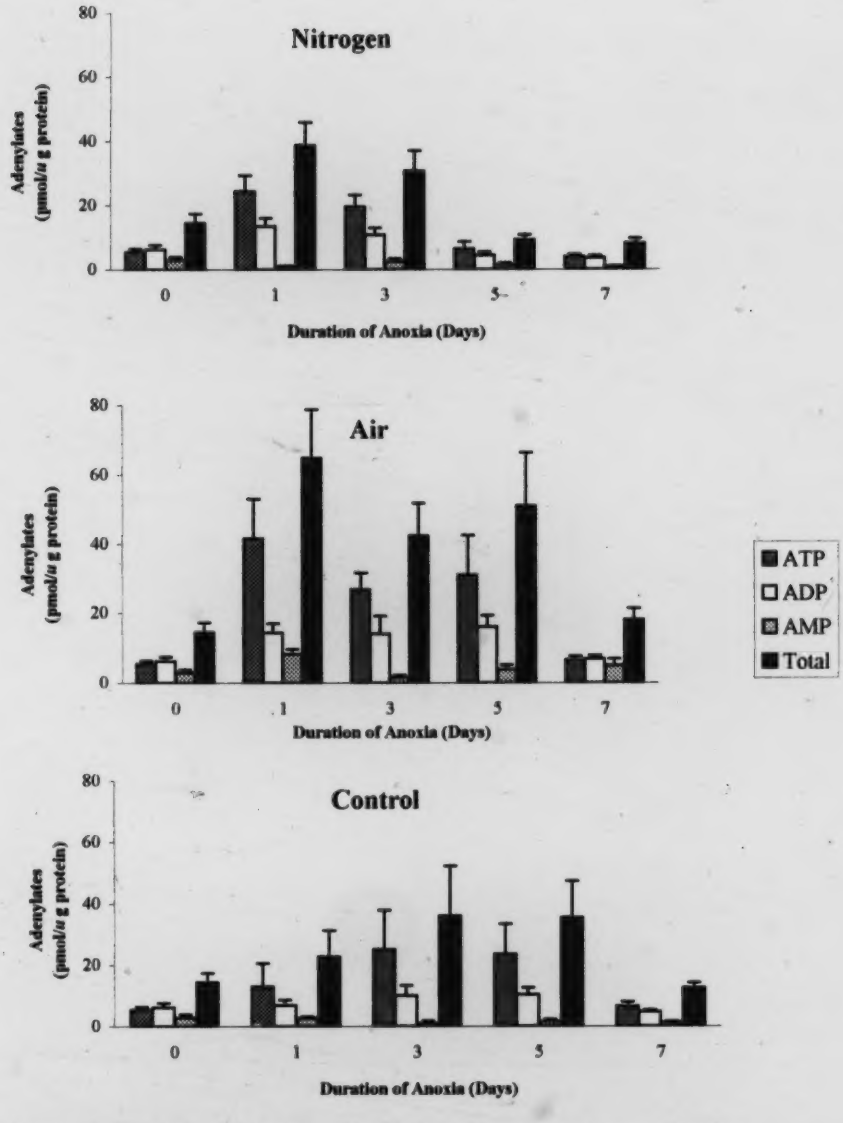


Figure 3-7. Adenylates in MDHEs after treatment in the Anoxia Culture System.

Microspore-derived haploid embryos were treated with either nitrogen gas or air in the Anoxia Culture System for up to seven days. Control MDHEs were grown in petri plates and shaken at 80 rpm. For each data point three independent experiments (of three samples of haploid embryos) were performed. The standard error of the mean is shown.

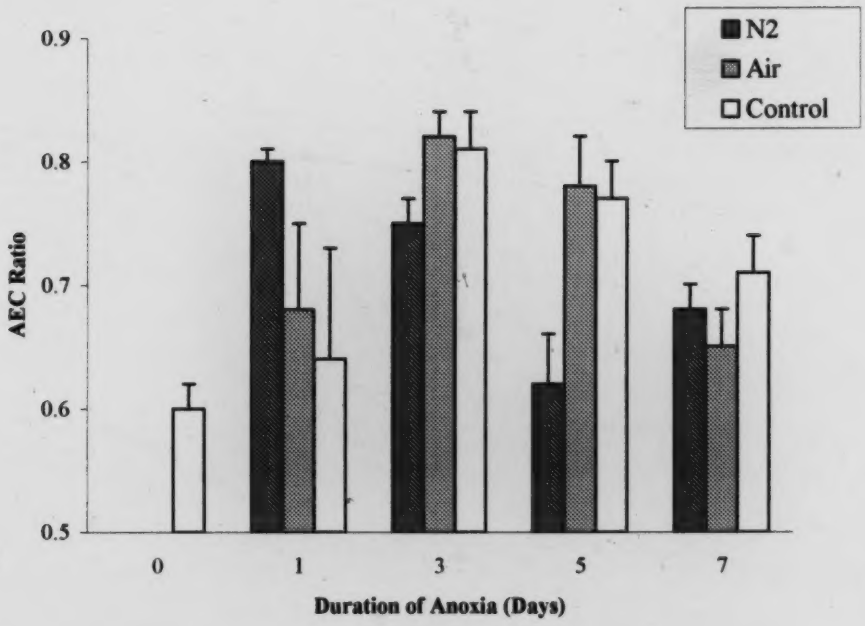


Figure 3-8. Adenylate Energy Charge ratio in MDHEs after treatment in the Anoxia Culture System

Microspore-derived haploid embryos were treated with either nitrogen gas or air in the Anoxia Culture System for up to seven days. Control MDHEs were grown in petri plates and shaken at 80 rpm. This analysis was performed over a seven day period, since the survival rate at nine days was less than 25%. For each data point three independent experiments (of three samples of haploid embryos) were performed. The standard error of the mean is shown.

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Chapter IV. Selection for Anoxia Tolerance using Microspore-Derived Haploid Embryos

Introduction

Any of our most important agricultural plants, including barley, maize, oats, soybeans and wheat tolerate even the most transient waterlogging poorly (Kennedy *et al.*, 1992). The anoxic response has been studied in many of these crop plants, for example, barley (Hanson and Jacobsen, 1984; Hanson *et al.*, 1984; Good and Crosby, 1989a; 1989b), maize (Wignarajah and Greenway, 1976; Sachs and Freeling, 1978; Sachs *et al.*, 1980; Roberts *et al.*, 1984a; 1984b; Hole *et al.*, 1992; Andrews *et al.*, 1993; 1994a; 1994b; Johnson *et al.*, 1994; Peschke and Sachs, 1994), soybeans (Brzezinski *et al.*, 1986; Russell *et al.*, 1990), winter wheat (Andrews and Pomeroy, 1989), and wheat (Thomson *et al.*, 1989; Thomson and Greenway, 1991; Waters *et al.*, 1991).

Although flooding is a significant agronomic problem, there are few reports of selection for flooding tolerance. Researchers have improved the flooding tolerance of crops such as rice (Eiguchi *et al.*, 1993; Karin *et al.*, 1982; Mackill *et al.*, 1993; Pushkaran and Balakrishna-Rao, 1985; Senadhira *et al.*, 1993; Yamauchi, 1992), corn (Khosravi and Anderson, 1990; Sachs and Jackson, 1993) and *Brassica rapa* (Daugherty and Musgrave, 1994) through selection at the seedling or whole plant stage. Flooding tolerant selections have also been made in wheat (Mian *et al.*, 1993), barley (Hamachi *et al.*, 1990), sorghum (Thseng and Hou, 1993), reed canary grass (Ivanov *et al.*, 1984) and sweet potato (Martin, 1983). The time and space costs of field-scale selection for plants with increased flooding tolerance may be one reason for the lack of interest in this area.

Although there are no reports of *in vitro* selection for flooding tolerance, plant tissue culture techniques have been used extensively in the past 30 years to select for tolerance to other environmental stresses. For example, *in vitro* selections have been made for salinity tolerance in rice (Vajrabhaya *et al.*, 1989; Heszky *et al.*, 1991; Vajrabhaya and Vajrabhaya, 1991; Sun *et al.*, 1991), maize (Lupotto *et al.*, 1994), wheat

(Karadimova and Djambova, 1993), and tomato (Hassan and Wilkens, 1988). Selections for tolerance to drought have been attempted in tomato (Bressan *et al.*, 1981; Handa *et al.*, 1983a) and tobacco cell cultures (Handa *et al.*, 1983b). Herbicide tolerance in maize has been selected for, *in vitro* (Marshall *et al.*, 1992; Sari-Gorla *et al.*, 1994; Frascaroli *et al.*, 1994). Low temperature tolerance has been selected for in potatoes utilizing pollen selection (Kristjansdottir, 1990) and in the ornamental African Violet plant, *Saintpaulia* (Grunewaldt, 1988). Tolerance to the pathogen *Fusarium* was selected for in wheat; in F_1 microspore culture populations (Fadel and Wenzel, 1993) and F_1-derived haploid sporophytes were utilized to select for tolerance to black shank in tobacco (Campbell and Wernsman, 1994).

In the *Brasssica* genus, somatic cultures have been used to select for tolerance to salinity in *Brassica juncea* (Jain *et al.*,1991; Kirti *et al.*, 1991) and canola (*Brassica napus* cv. 'Westar'). As well, they have been utilized for selection for salinity tolerance in chinese cabbage (*B. campestris*) (Chandler *et al.*, 1988) and in 11 cultivars of oilseed *Brassicas* (*B. juncea*, *B. campestris*, and *B. carinata*) (Tyagi and Rangaswamy, 1993). Selections have also been made successfully from gametophytic cultures. Anther or microspore-derived haploid embryo (MDHE) culture, or pollen selection has been used in *Brassica napus*, to select for resistance to the herbicides, chlorosulfuron (Ahmad *et al.*, 1991), and the sulfonylureas (Swanson *et al.*, 1988), and fungal pathogens such as *Alternaria brassicicola* (Ahmad *et al.*, 1991), and *Phoma lingam* (Sacristan, 1982). The utility of selection at the male gametophyte stage is based upon the assumption that many sporophytic genes are expressed in the gametophyte as well. In addition, plant regeneration may be less difficult from selections done with the gametophyte and the selected traits are more likely to be genetically inherited (Dougall, 1980).

To make a successful selection, there must be variation within the population for the trait of interest. This variation may be naturally occurring, or may arise from mutagenic treatment. Natural variation for anaerobic tolerance exists in maize. Lemke-Keyes and Sachs (1989) characterized an Anaerobic Tolerant Null (ATN) mutant which

lacked ADH1 enzyme activity, but was able to survive 24 hours of anaerobic treatment. The mutation was identified through crosses with a double ADH1 null mutant as a single recessive locus. ADH1 null mutants which lacked the ATN factor could survive only a few hours of anoxia.

Somaclonal or gametoclonal variations, which may arise after one or more cycles of tissue culture, are another source of phenotypic variation (Larkin and Scowcroft, 1981). Although the MDHEs do not pass through an undifferentiated state, *per se*, there has been an alteration of the normal developmental pathway to pollen formation. This in itself may be sufficient to generate variation. Somaclonal variation has been utilized in the improvement of sugar cane (Larkin and Scowcroft, 1981), potato cultivars (Larkin and Scowcroft, 1981; Ball and Seilleur, 1986), tobacco (Larkin and Scowcroft, 1981; Gabard *et al.*, 1986; Marion-Poll *et al.*, 1986) and in ornamentals (Buiatti *et al.*, 1986; Bouharmont and Dabin, 1986).

The objective of this study was to determine if the MDHE system could be used to select, *in vitro,* for tolerance to anoxia and whether the selection would be stable and heritable. A selection was made from the T4079 MDHE population using the Anoxia Culture System and fertile, diploid seed was produced for characterization. The putative anoxia-surviving (AS) material was compared to the parental material and another related line for tolerance at the level of the MDHEs. The AS line was backcrossed in reciprocal combinations to the parental material (T4079) to determine the possible pattern of inheritance of the anoxia-tolerant trait and evaluate the anoxia-tolerance of F_1 progeny at the MDHE stage. The anoxia tolerance of the F_1 progeny was compared with that of the parental material. The F_1 progeny lines were backcrossed to AS_2 and T4079 in reciprocal combinations. As well, AS_3, T4079 and the F_1 progeny lines were self-pollinated. Finally, the extent of anoxia tolerance in etiolated seedlings of the AS_3 line was assessed. The objective was to test the progeny of the backcrossed and self-pollinated plants at the seedling stage, but the high degree of variability in survival made it impossible.

Materials and Methods

Plant Growth Conditions

(See Chapter II). The original seed was obtained from Dr. Wilf Keller, Plant Biotechnology Institute, Saskatoon Saskatchewan (T4079) and from Dr. Rachael Scarth, Department of Plant Science, University of Manitoba, Winnipeg, Manitoba (R8311). Plants were self-pollinated for the purpose of seed increase. These two lines were chosen because of their utility for MDHE production. The line T4079 is from the cultivar Topas and R8311 was derived from the cultivar Ariel (Dr. Rachael Scarth, personal communication). The AS line was selected from a population of T4079 at the MDHE stage under anoxic treatment conditions.

Self-Pollination of Plants for Seed Production

(See Chapter II)

Microspore-Derived Haploid Embryo Culture

(See Chapter III)

Microspore-Derived Haploid Embryo Treatment in the Anoxia Culture System

Microspore-derived haploid embryos were treated with either nitrogen gas or air as in Chapter III. The nature of the MDHE system resulted in several problems in terms of testing the various lines and generations of material at the same time. All the survival tests had to be performed on fresh MDHE cultures at the late torpedo to very early cotyledonary stage. It was impossible to ensure that all the lines which were being cultured would result in MDHEs at the correct stage at the right time to run a properly controlled, fully replicated series of tests. In order to control for the factor of time in these experiments, every care was taken to repeat the tests in an identical fashion. It is for this reason that no inferential statistics have been used to analyze this data.

Survival Assessment of the MDHEs

(See Chapter III)

Selection for Anoxia-Tolerance and Plant Regeneration

The cultures of MDHEs were subjected to anoxia treatments in the Anoxia Culture System, as previously described (Chapter III). During the initial screening for anoxia tolerance, a total of approximately 2970 MDHEs were treated with nitrogen gas and 2810 were treated with air. One MDHE which had survived 10 days of anoxia treatment was selected from the T4079 population (designated: AS_1). The developing plantlet was subcultured to Regeneration Media under moderate light levels (100 *u*mol m^{-2} s^{-1}, 16 hour photoperiod, 25°C) for the next six months. After the AS_1 plantlet had developed shoots, leaves and roots, it was potted in sterile potting mix (as above) and moved to a misting chamber, under illumination (100 *u*mol m^{-2} s^{-1}, 16 hour photoperiod, 25°C) for approximately two months. Once the plantlet had become successfully established it was moved to a light bench (100 *u*mol m^{-2} s^{-1}, 16 hour photoperiod, 25°C) where it bolted and produced a few small flowers on one of the shoots. It was observed that the AS_1 plantlet had undergone spontaneous diploidization at some time during the isolation, treatment or regeneration processes. The seeds were very small and shrunken and did not germinate.

Clonal Propagation of the Putative Anoxia Insensitive Plantlet

Five cuttings, approximately 5-7 cm long, each with several leaves, were taken from lateral branches of the AS_1 plant. The distal tips of the cuttings were treated with 0.1% indole-3-butyric acid (Stim-Root #1, Plant Products Co. Ltd.). The cuttings were placed in sterile peat pellets and placed on a light bench (100 *u*mol m^{-2} s^{-1}, 16 hour photoperiod, 25°C). From the five cuttings taken, three developed roots and survived. After two months the cuttings were transferred to sterile potting mixture (as above) and placed in an environmental chamber (250 *u*mol m^{-2} s^{-1}, 16 hour photoperiod, 20°C/15°C (light/dark)). These plants were self-pollinated to produce second generation, AS_2 seed. Later, plants grown from AS_2 seed were self-pollinated, producing the third generation (AS_3) seed.

Crossing and Backcrossing

The plants (AS_2 and T4079) were grown as above until flowering. Before crossing, any open flowers on the inflorescence of the maternal parent were removed. The sepals, petals and anthers of the unopened buds (bud size: 0.5-1.0 cm) of the maternal parent were carefully removed to expose the stigma. Freshly dehisced anthers with pollen were collected from open flowers on the paternal parent using fine forceps. The pollen was gently brushed onto the stigmatal surface of the maternal parent. After cross-pollination was complete, the floral meristem of each inflorescence was removed and the inflorescence was bagged as above. Growing conditions and seed collection were as above. Four reciprocal crosses were done between the AS_2 line and the T4079 parental line. Four of the F_1 progeny lines (AT_4, AT_3, TA_4 and TA_3) from the cross were backcrossed in reciprocal combinations to both the parental lines (T4079 and AS_3). As well, the parental lines and the F_1 lines were self-pollinated. The self-pollination procedure was the same as that used for seed production.

Anoxic Treatment of Etiolated Seedlings

Seeds of AS_3 or T4079 were surface sterilized in 1.2% sodium hypochlorite (with two drops of Tween-20 as a surfactant) for 20 minutes, followed by three rinses (five minutes each) in sterile, deionized water. The seeds were germinated in glass petri plates containing sterilized perlite, in darkness at 25°C, for three to four days. The perlite was maintained moist, but not wet, by a filter paper wick in a tray of half-strength Hoagland's solution (Wetter and Constabel, 1982). Etiolated seedlings were selected at the cotyledonary stage, at the time of hypocotyl hook straightening (three to four days old). Seedlings were individually inserted into foam plugs and placed into Styrofoam rafts floating in half-strength Hoagland's solution in rectangular, polyethylene containers with air-tight lids ('Super Seal' brand, 5.6 L). Treatment gas was supplied to each container through a polyvinyl chloride tubing line (Nalgene Co., Fisher Scientific Ltd.) from the gas cylinders, through a small hole, to a 15 cm aerator stone in the nutrient solution. The roots of the seedlings were suspended in the nutrient solution that was sparged continuously with either nitrogen gas (prepurified, minimum purity: 99.998% N_2, Linde,

Union Carbide) or air (extra dry), depending on the treatment. A fermentation lock inserted through the lid of the containers allowed the treatment gas to escape, while preventing contamination. Both holes were sealed with 'Hold It' (Dixon Ticonderoga Co.) to prevent any other air from entering or exiting. During the period of anoxia treatment, the containers were tightly closed and were covered with black plastic, and the lights were turned off in the environmental chamber (20°C). The seedlings were treated for periods of time from 18 to 72 hours. Each data point was the mean of three independent experiments, consisting of two samples of five seedlings each (n=3), except for the 24 hour treatment, which was replicated eight times (n=8) The sample means are shown with the standard error of the means.

Assessment of Seedling Survival after Anoxia Treatment

The assessment of seedling survival was performed in a environmental chamber (120 μmol m^{-2} s^{-1}, 16 hr photoperiod, 20°C). After being treated for the specified period of time, the lids of the tubs were removed and the solution in both the tubs were sparged with a continuous flow of air (as above). After three days, the survival of the seedlings was evaluated according how they responded to the anoxia stress. A preliminary study had determined that only Group I seedlings would survive for a further 14 days. Group I seedlings were therefore assessed as survivors.

Response Groups:

I. The cotyledons are both greened and growing and the roots are growing.

II. The cotyledons are either both partially green, part yellow or one is green and the other is brownish or yellowed. The apical meristem does not appear to be dead.

III. The cotyledons are brown or yellow and shriveled up, and the apical meristem is dead. There is no evidence of root growth.

Results and Discussion

The goal of this work was to determine whether *in vitro* selection for anoxia tolerance could be made in *Brassica napus* from cultures of MDHEs. The MDHE cultures were subjected to anoxia treatments in the Anoxia Culture System, as previously described (Chapter III). During the initial screening for anoxia tolerance, a total of approximately 2970 MDHEs were treated with nitrogen gas and 2810 were treated with air (Table 4-1). For each time point (except for day 11), three independent experiments were performed, consisting of one sample each (n=3). Each sample consisted of one flask with approximately 100 to 200 MDHEs. The 11 day treatment was only replicated twice (pseudoreplication) because of the inherent problems of maintaining sterility over such a long treatment. As a result, inferential statisitics were not performed on this data. One MDHE, which had survived 10 days of anoxia treatment, was selected from the T4079 population. The putative anoxia-tolerant MDHE was regenerated to a whole plant. Once large enough, the putative anoxia-tolerant plant (designated: AS_1) was clonally propagated from stem cuttings and plants were regenerated. It was observed that the AS_1 plantlet had undergone spontaneous diploidization at some time during the isolation, treatment or regeneration processes. This conclusion was based upon the observation that the AS_1 plants produced inflorescences with normal flowers, which, upon self-pollinization produced viable seed. The AS_1 (first generation) plants were self-pollinated to produce AS_2 (second generation) seed.

The AS_2 plants appeared normal in comparison to T4079 and R8311 plants at the seedling and flowering stages (Figures 4-1a and 4-1b). Microspore-derived haploid embryos produced from AS_2 plants had an unusual, fused cotyledon phenotype at later stages of development in comparison to MDHEs of R8311 (Figure 4-2a and 4-2b). As the R8311 was growing at the same time, a photograph of it is shown to illustrate the normal phenotype seen in both lines R8311 and T4079. The unusual, fused cotyledon phenotype was not observed in the MDHE cultures from subsequent generations, so it may have been an artifact of the regeneration process from tissue culture. Amhad and co-workers (1991) also observed cotyledon and root developmental variants (<5%) in the

MDHE cultures of *B. napus* L. ssp. oleifera, but the nature of this variation was not specified.

The anoxia tolerance of AS_2 MDHEs was tested against that of the line T4079 (Figure 4-3) over periods of 9 and 11 days treatment with nitrogen gas in the Anoxia Culture System. Microspore-derived haploid embryos from AS_2 plants showed a small improvement over T4079. After nine days anoxia treatment, approximately 43.5% of the AS_2 MDHEs survived compared to 15.7% of the T4079. After 11 days of anoxia treatment, 3.1% of the AS_2 MDHEs survived, while none of the T4079 embryos survived. It is possible this difference may attributed to usually high survival rates in this test only, since it was not possible to include a control for time. These results are approximately the same as noted previously (Chapter III) and again indicate that there is a high degree of variability in the survival of the haploid embryos after treatment with anoxia. The variable nature of microspore cultures and haploid embryos have been observed in other studies. There was up to 40% variation in the survival of microspores of *B. napus* after irradiation with ultraviolet light (Ahmad *et al.*, 1991). The presence of this variation in the haploid embryo populations should make it possible to select from the population for tolerance to anoxia. The results from these preliminary tests indicated that there was sufficient improvement to continue with the characterization of the AS line.

Figure 4-4a shows a representative sample of MDHEs, treated for nine days, after three days on Regeneration Media. The cotyledons and hypocotyls of the control samples (treated with air) (Figures 4-4a and 4-4b) turned green and there was evidence of primary and lateral root development (Response Group I). The cotyledons and hypocotyls of the anoxia-treated R8311 MDHEs were white or brown and there was no evidence of greening (Response Group III and IV). In contrast, the anoxia-treated AS_2 MDHEs showed some greening in the cotyledons and hypocotyls, as well as primary and lateral root growth. These MDHEs would be assessed as Response Group I and II. The photo of the R8311 line was used here to illustrate the response seen in both lines R8311 and T4079, in comparison to the AS_2 line.

Based on the results of the preliminary tests, it was felt that the AS_2 line demonstrated a small improvement in anoxia tolerance. Crosses were made in reciprocal combinations between the AS_2 plants and the wild type *B. napus*, T4079 parental material, to determine the pattern of inheritance of the anoxia-insensitive trait (Figure 4-5). Four F_1 lines (AT_3, TA_3, AT_4, TA_4) of plants were produced and MDHEs were produced from them. The anoxia tolerance of the F_1 MDHEs was evaluated after 9, 10 and 11 days of anoxia treatment (Figure 4-6). This figure shows the average response of the four F_1 lines, from a number of different tests. It was very difficult to ensure that the MDHEs from all six lines were at the same developmental stage at the same time, so the four F_1 lines were only tested together once, but the individual lines were tested with appropriate controls in separate tests.

The nature of the MDHE system resulted in several challenges in terms of testing the various lines and generations of material at the same time. All the survival tests had to be performed on fresh MDHE cultures at the late torpedo to very early cotyledonary stage. It was impossible to ensure that all the lines which were being cultured would result in MDHEs at the correct stage at the right time to run a properly controlled, fully replicated series of tests. As well, the course of these selections, crosses and tests extended over more than five years, which further complicated the situation. The regeneration and propagation of the original selection, the putative anoxia tolerant MDHE took almost three years alone. In order to control for the factor of time in these experiments, every care was taken to repeat the tests in an identical fashion. It would have been advantageous to have a common control which could have been included in the various different survival tests, but that was not possible, based upon the MDHE system. It is for this reason that no inferential statistics have been used to analyze this data.

Three of the four F_1 lines (TA_3, TA_4 and AT_4) showed higher levels of anoxia tolerance compared to the parental material, T4079 (Figure 4-3). After nine days anoxia

treatment, only 15.7% of the T4079 embryos survived, compared to 63% (TA_3), 81% (AT_4) and 77% (TA_4). One of the lines, AT_3 , had consistently lower survival rates (~ 26%) than did the other three. The results in three of the four lines indicated that it was possible to improve the anoxia tolerance at the MDHEs stage through screening *in vitro*, and that this trait had been stably inherited. The relatively high survival of three of the four F_1 lines (TA_3, AT_4 and TA_4), compared to the parental lines may be a result of hybrid vigour.

The four F_1 lines were backcrossed in reciprocal combinations with both the parents; T4079 and AS_3 plants (Figure 4-5). As well, all four of the F_1 plants were self-pollinated, as were the AS_3 and T4079 plants. The goal was to screen the progeny from the backcrosses and self-pollinations for survival at the seedling stage to avoid the complications of screening at the MDHE stage. To this end, at least 20 siliques from each cross and self-pollination were obtained in order to have approximately 200 seeds of each.

Prior to the evaluation of the backcrossed and self-pollinated lines (#1- #24), I re-examined the MDHE stage in the AS_3 and T4079 lines (Figure 4-7). After nine days treatment in the Anoxia Culture System, it appeared there was no difference between the anoxia tolerance of the AS_3 line and T4079. There also had been no improvement in the anoxia tolerance at the level of the MDHEs, in comparison to the initial screening.

Since the goal was to screen the backcrossed and self-pollinated lines at the seedling stage, I examined the anoxia tolerance of etiolated seedlings of the lines AS_3 and T4079 (Figure 4-8). After 48 and 72 hours, there was consistently no survival of the seedlings after anoxia treatment, in either of the lines. Extensive screening (n=16) of the etiolated seedlings at 24 hours of anoxia indicated that there was a wide degree of variability in the response to anoxia treatment, with no consistent differences in the response of the two lines. Based upon these results, it was decided not to pursue the analysis of the anoxia tolerance of the backcrossed and self-pollinated lines (#1- #24).

It appears that despite selection, the AS_3 population still varies in its ability to tolerate anoxia at the level of MDHEs and etiolated seedlings. This indicates that there was not a stable genetic basis of flooding tolerance in the AS line, but that there may be an environmental or developmental factor or factors responsible for the survival of the material under long periods of anoxia. Developmental or epigenetic change is a source of phenotypic variation in tissue cultures that can either occur spontaneously or as a result of specific environmental conditions (Binns, 1981). Epigenetic effects are defined as heritable changes in cytoplasmic or nuclear genetic elements other than coding genes. Epigenetic changes arise from the stable alterations in the expression of the genome and are distinguished from random genetic change by the following criteria: they are (1) directed and may occur regularly, (2) stable and heritable at the cellular level, (3) potentially reversible, (4) restricted to the genetic potential of the cell (Binns, 1981).

When selecting for tolerance to an environmental stress in plant tissue cultures, true selection of tolerant phenotypes may be confused with selection of adapted cells or plants. Possibly, during the selection of anoxia-tolerant MDHEs, some of the genotypes in the culture adapted to the stress environment, but this ability was lost in the subsequent generations.

Alternatively, somaclonal or gametoclonal variation may have resulted in the anoxia-tolerant MDHEs. At one time, it was assumed that all the plants arising from somatic plant tissue culture should be exact copies of the parental plant. Phenotypic variants have frequently been observed amongst the plants regenerated from culture (Larkin and Scowcroft, 1981). The term 'somaclonal variation' was coined by Larkin and Scowcroft (1981) to describe the phenotypic variation observed in plant material after one or more cycles of tissue culture. This was used as a tool for the production of variants of interest for a number of years, however, it was later discovered that the variants were not stable over subsequent generations. Somaclonal and gametoclonal variation appears not to be species or organ-specific and variation among somaclones has been observed for a

wide array of characters. The lack of stability of such variation has brought the use of somaclonal variants for selection into question.

In retrospect, there are a number of things that probably could have been done differently in order to obtain an anoxia-tolerant line. It may have been better to continue the selection until there was a population of tolerant or resistant MDHEs in culture, instead of using only one plantlet to create the line. If this was not forthcoming, chemical mutagenesis could have been employed to increase the rate of mutations. Plants regenerated from this tolerant population of MDHEs could have been used for several repeated cycles of cross-pollination and production of MDHEs, with subsequent cycles of selection pressure.

In addition, it was concluded that the MDHE system is probably not suitable for the selection, *in vitro*, for tolerance to anoxia. The demands of the system prevented a properly controlled, fully replicated series of tests. It would have been advantageous to have a common control which could have been included in the various different survival tests, to control for the time variable between experiments. In addition, the MDHEs in culture are already experiencing a degree of hypoxia, which may have made it more difficult to select for anoxia tolerance.

Figure 4-1a. AS_2 (labelled 'Aim'), T4079 and R8311 plants at seedling stage (three to four weeks old).

Plants were grown in 20 cm pots in a peat:sand:vermiculite (2:1:2) mixture in an environmental chamber for four to five weeks. Upon the onset of flowering, the plants were transferred to 10°C/7°C under the same light and photoperiod regime to continue growth.

Figure 4-1b. AS_2 (labelled 'Aim'), T4079 and R8311 plants at flowering (six to eight weeks old).

a

b

Figure 4-2a. R8311 MDHEs on Regeneration Media, showing normal cotyledon phenotype.

After treatment in the Anoxia Culture System for nine days, the MDHEs were removed from the flasks and plated on Regeneration Media. The petri plates were put onto a light bench (100 *u*mol m^{-2} s^{-1}, 16 hour photoperiod, 25°C) and after three days, the survival of the MDHEs was evaluated. The R8311 MDHEs exhibit normal cotyledonary phenotype, as compared to the AS_2 MDHEs, which have fused cotyledons. This unusual phenotype was not observed in subsequent generations.

Figure 4-2b. AS_2 MDHEs on Regeneration Media, showing fused cotyledon phenotype.

a
1cm

b
1cm

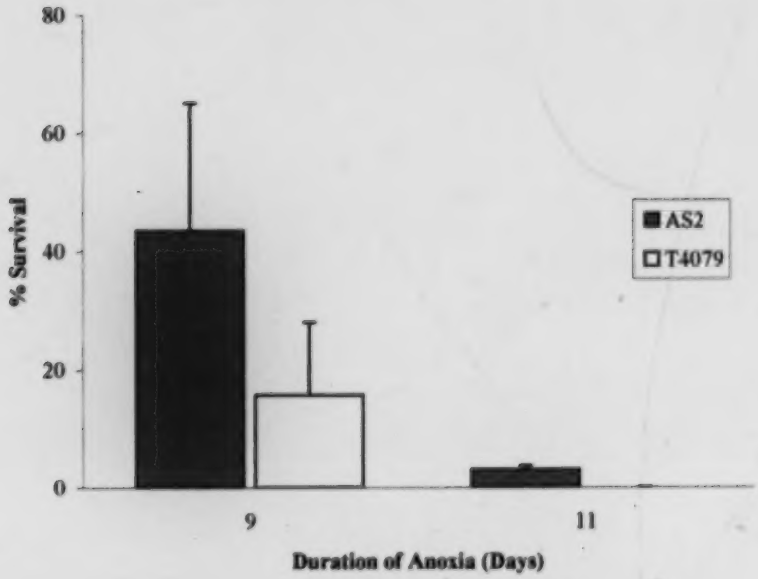

Figure 4-3. Survival of AS_2 and T4079 MDHEs after treatment in the Anoxia Culture System.

Microspore-derived haploid embryos of AS_2 and T4079 were treated for nine or eleven days with nitrogen gas in the Anoxia Culture System. The control MDHEs (treated with air) survived to 83% (+/-10%). Each data point is the mean of three independent experiments of two samples each (n=3), with the standard error of the means.

Figure 4-4a. R8311 MDHEs, after nine days treatment in the Anoxia Culture System and three days on Regeneration Media.

The MDHEs were plated on Regeneration Media after treatment in the Anoxia Culture System with either nitrogen gas or air for nine days. The petri plates were placed a light bench for three days, and the survival of the embryos was evaluated on the basis of the their response to anoxia.

Response Groups:

I- Cotyledons greened, primary root growing, many lateral roots.
II- Cotyledons white, hypocotyl developing callus and greening, primary root growing, many lateral roots.
III- Cotyledons white, no callus growth or greening in hypocotyl region, primary root growing, no lateral roots.
IV- No growth or greening, embryos dark-colored.

Figure 4-4b. AS_2 MDHEs (labelled 'Aim') after nine days treatment in the Anoxia Culture System and three days on Regeneration Media.

R8311 9 days Air
R8311 9 days N_2
a

AIM 9 days Air
AIM 9 days N_2
b

Figure 4-5. Diagram of crosses and backcrosses.

Second generation Aim (AS_2) plants were cross-pollinated in reciprocal combinations to T4079 plants. Four of the F_1 progeny lines (AT_3, AT_4, TA_3 and TA_4) were subsequently backcrossed in reciprocal combinations to both the parental lines (AS_3 and T4079). As well, the AS_3 and T4079 lines and the F_1 lines were self-pollinated.

First Generation AS_1	Regenerated plantlet and clonal propagants.	-Selfed $\rightarrow AS_2$
Second Generation AS_2	-Preliminary assessment of anoxia tolerance in MDHEs. (Figures 4-1, 4-2, 4-3, 4-4)	-Selfed $\rightarrow AS_3$ -Crossed with T4079 $\rightarrow$ F_1 lines (AT_3, TA_3, TA_4, AT_4)
Third Generation AS_3	-Assessment of anoxia tolerance in MDHEs and etiolated seedlings. (Figure 4-7, 4-8)	-Backcrossed to F1 lines $\rightarrow$ BC Lines (#1-#8) -Selfed $\rightarrow$ Selfed Lines (#17, 18)

Original Cross

	AS2 x T4079	T4079 x AS2
	↓	↓
F1 Lines:	AT3, AT4	TA3, TA4

Backcrosses

TA4 X AS3- 1	AS3- 1 X TA4	AT3 X AS3- 1	AS3- 1 X AT3
↓	↓	↓	↓
#1	#2	#3	#4
AT4 X AS3- 2	AS3- 2 X AT4	TA3 X AS3- 2	AS3- 2 X TA3
↓	↓	↓	↓
#5	#6	#7	#8
TA4 X T4079-1	T4079-1 X TA4	AT3 X T4079-1	T4079-1 X AT3
↓	↓	↓	↓
#9	#10	#11	#12
AT4 X T4079-2	T4079-2 X AT4	TA3 X T4079-2	T4079-2 X TA3
↓	↓	↓	↓
#13	#14	#15	#16

Selfed Lines

AS3-1	AS3-2	T4079-1	T4079-2	TA4	AT4	TA3	AT3
⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗
↓	↓	↓	↓	↓	↓	↓	↓
#17	#18	#19	#20	#21	#22	#23	#24

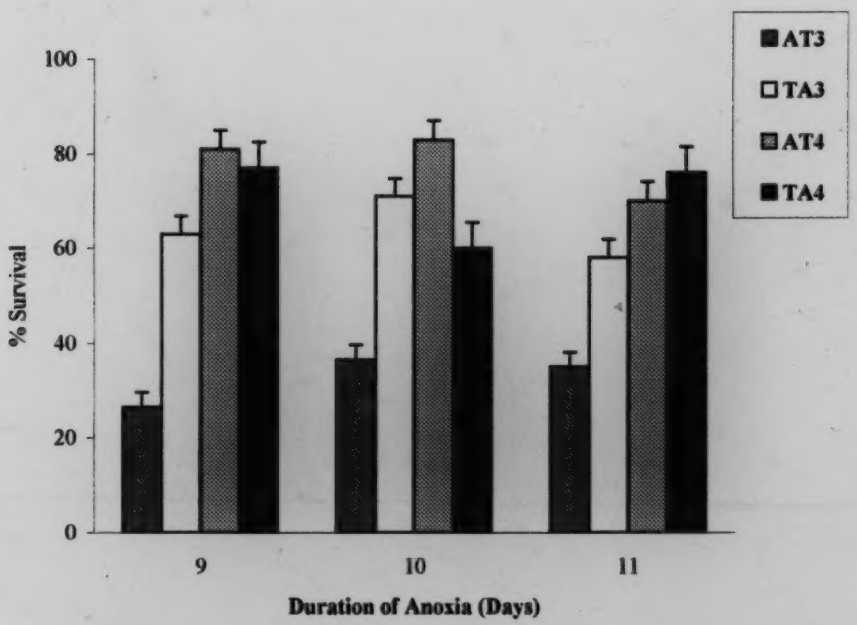

Figure 4-6. Survival of F_1 MDHEs after treatment in the Anoxia Culture System.

Microspore-derived haploid embyos from the F_1 generation were treated for periods of 9, 10 and 11 days in the Anoxia Culture System. Each data point is the mean of three independent experiments of two samples each (n=3), with the standard error of the mean.

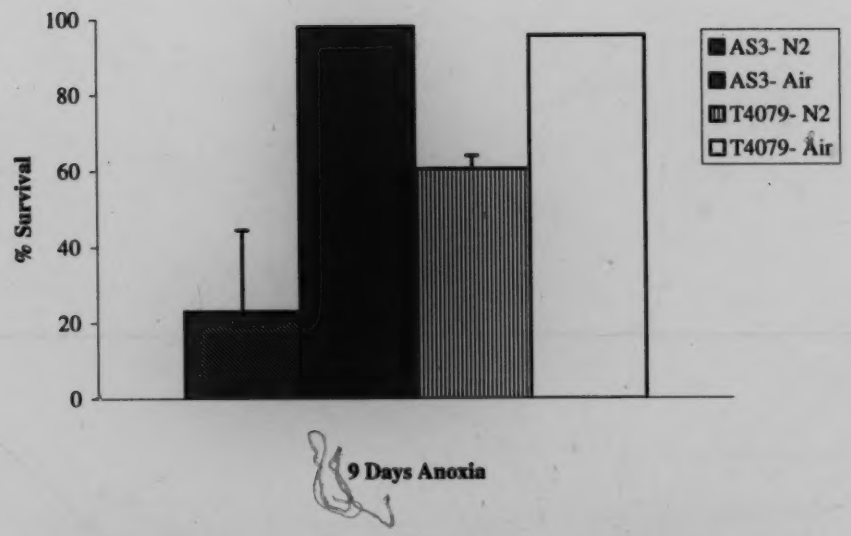

Figure 4-7. Survival of AS_3 and T4079 MDHEs after nine days treatment in the Anoxia Culture System.

Microspore-derived haploid embyos of AS_3 and T4079 were treated with nitrogen gas or air for nine days in the Anoxia Culture System. Each data point was the mean of three independent experiments with two samples each (n=3), with the standard error of the means.

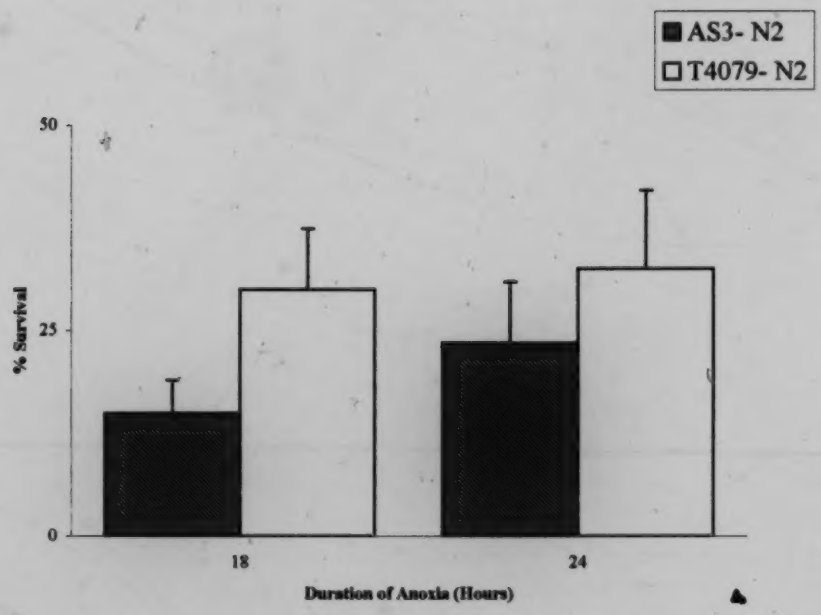

Figure 4-8. Survival of etiolated seedlings of AS_3 after treatment in anoxia or air.

Etiolated seedlings (three to four days old) were treated in closed containers, in the dark, with either nitrogen gas (anoxia) or air for 18 to 72 hours. There was 0% survival in both lines after 48 hours and 100% of the air-treated seedlings survived. Each data point was the mean of at least three independent experiments with two samples each (n=3). The 24 hour treatment was tested eight times (n=8). The mean survival of the etiolated seedlings is shown, with the standard error of the means.

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Chapter V.

The Anaerobic Response during Seed Germination and Early Seedling Growth

Introduction

Seed germination begins with water uptake by the seed (imbibition) and ends with the start of elongation by the embryonic axis, usually resulting in radicle emergence from the seed coat (Bewley and Black, 1994). Germination, then, in *senso stricto*, does not include seedling growth, which commences when germination finishes. Many studies on the metabolism of germinating seeds do not indicate the time of radicle protrusion, and thus may actually represent changes occurring during seedling development (Botha *et al.*, 1992).

Germination occurs in most seeds in three phases, which follow a sigmoidal pattern. Phase I, imbibition of the seed, is characterized by a steep initial rate of water uptake. This is largely a consequence of matric forces and occurs regardless of whether the seed is dormant, viable or nonviable. The second phase is the lag phase of water uptake. During this phase major metabolic events take place in preparation for radicle emergence. During phase III there is another steep rise in water uptake that is related to the changes occurring in the cells of the radicle as they extend. Once the radicle has penetrated the testa, mitosis occurs in the apical region of the radicle and there is a rapid increase in the number of cells (Bewley and Black, 1994).

There is general agreement that in many seeds there is enhanced enthanolic fermentation during germination, the seeds produce substantial quantities of ethanol and there is a delay in O_2 consumption (Botha *et al.*, 1992). In some seeds it is believed that the lag phase in O_2 consumption occurs as a result of partially anaerobic conditions. Seed coats or other surrounding structures may limit O_2 uptake to the imbibed embryo or storage tissues (Bewley and Black, 1994). Even under well-aerated conditions, ethanol

and lactate accumulation may occur in imbibed seeds, indicating a 'natural anaerobiosis' (Raymond *et al.*, 1985). Alternatively, if there was more rapid activation of glycolytic enzymes than the development of the mitochondria, there would be a lag in O_2 uptake. An accumulation of pyruvate might be diverted temporarily to the fermentative pathway. This would account for high levels of alcohol dehydrogenase (ADH) activity and the accumulation of ethanol in germinating seeds in the absence of a true anaerobic stress situation. This was observed in mung beans by Morohashi and Shimokoriyama (1972; 1975). Leblova (1978) found that in a wide variety of seeds, ADH activity increased in the first few hours of germination, reached a characteristic maximum after one to two days (depending upon the type of seed), and then decreased.

Our previous work suggested that ADH activity may change with development (Chapter II and III). This has also been observed in young seedlings of maize (VanToai, *et al.*, 1995), where two day-old seedlings were found to be much more flooding tolerant than three day-old ones. There were indications that ADH activity did not correlate with the quantity of the enzyme. Further, it was observed in the MDHEs that a high level of ADH activity did not necessarily confer survival. Thus, the objectives of this study were to characterize the pattern of germination and the activity of ADH during germination and early seedling development. As well, the patterns of ADH isozyme expression were determined and the effects of anoxia during seed germination and early seedling growth were examined.

Materials and Methods

Seed Source

The original seed was obtained from Dr. Rachael Scarth, Department of Plant Science, University of Manitoba, Winnipeg, Manitoba (R8311). Plants were self-pollinated for the purpose of seed increase. The line R8311 was derived from the cultivar Ariel (Dr. Rachael Scarth, personal communication).

Self-Pollination of Plants for Seed Production

(See Chapter II)

Seed Germination

Seeds of R8311 were surface sterilized in 1.2% sodium hypochlorite (with two drops of Tween-20 as a surfactant) for 20 minutes, followed by three rinses (five minutes each) in sterile, deionized water. The seeds were germinated in glass petri plates containing sterilized perlite, in a environmental chamber (120 *u*mol m^{-2} s^{-1}, 16 hour photoperiod, 22°C). The perlite was maintained moist, but not wet, by a filter paper wick in a tray of half-strength Hoagland's solution (Wetter and Constabel, 1982).

Native Gel of ADH isozymes

Non-denaturing PAGE of isozymes of ADH was performed according to Hanson and Jacobsen (1984). Seeds were germinated as above. Barley seedlings (three to four days old) were treated in the Hydroponic Treatment System for four days with either nitrogen gas or air and were included as a comparison. Samples were ground in 500 *u*L sample buffer (150 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol) in 1.5 mL microcentrifuge tubes using a Pellet Pestle (Kontes). The crude homogenates were microcentrifuged for five minutes at 4°C at 13,000 rpm (~ 6000 x g). Aliquots containing 50 *u*g protein (except for the barley seedling roots: 25 *u*g) (with 15% glycerol and 0.01% bromophenol blue) were run on a 1.5 mm, 7.5% non-denaturing polyacrylamide gel (7.4% acrylamide- 0.1% bis, with 0.38 M Tris-HCl (pH 8.5)) on a Bio-Rad large gel apparatus. The gel was polymerized with 0.08% TEMED and 0.05% APS. The stacking gel contained 4.0% acrylamide-0.63% bis, 75 mM Tris-HCl (pH 8.5), 20% sucrose, polymerized with 0.06% TEMED and 0.05% APS. The gels were run at 4°C, with Laemmli running buffer at 10 mA/gel until the dye front entered the running gel, then at 20 mA/gel for the remainder of the run. The gels were stained as in Chapter II.

ADH Enzyme Assays

(See Chapter II)

Western Blots

An equal amount of total buffer-soluble protein (10 *u*g) was separated in Laemmli running buffer (25 mM Tris-190 mM glycine (pH 8.5)) (Laemmli, 1970) on a 12% SDS polyacrylamide gel (stacking gel: 4%) at 180 volts (constant voltage) using a Bio-Rad Minigel apparatus (See Chapter II).

Seedling Treatment in the Hydroponic Treatment System

Seedlings (two to four days old) (25 per treatment) were removed from the perlite and individually inserted into foam plugs and placed into Styrofoam rafts floating in half-strength Hoagland's solution (Wetter and Constabel, 1982) in rectangular, polyethylene containers (4.0 L, 14 cm by 40 cm) (See Chapter II).

Early Seedling Growth under Anoxia

Seedlings were removed from the flasks and immediately ground in grind buffer for ADH assays (as above) (See Chapter III).

Results and Discussion

The objective of this study was to study the anaerobic response of germinating seeds. The time course of seed germination was characterized, following water uptake by the seeds and radicle emergence in the first 32 hours postimbibition (Figure 5-1). The germination pattern followed a sigmoidal pattern described by Bewley and Black (1994). During the the initial phase of germination, the first six to eight hours, there was a steep rise in seed weight, as imbibition occurred. Between approximately eight and 21 hours, the rate of water uptake slowed in preparation for germination and radicle protrusion. After the emergence of the radicle, between 18 and 24 hours, there was again a steep rise in water uptake and the seedlings increased in weight. Figure 5-2 illustrates the stages of seedling growth from one day (1-3 mm radicle protrusion) to four days postimbibition. After two days, the seed coat has been shed and the cotyledons are greening. Around

three days, the hypocotyl hook straightens and the cotyledons are greened. After four days, the hypocotyl elongates and the primary root has grown as well.

Changes in alcohol dehydrogenase (ADH) enzyme activity were followed over the four day period of seedling development (Figure 5-3). There was a substantial amount of ADH activity in the dry seeds, which increased slightly after 24 hours (end of phase II). ADH activity was very high (1.5 *u*mol/min/mg protein) in radicles of the germinated seeds (24 hours) compared to the whole seed after 24 hours. This difference may be explained in part by the presence of large amounts of storage proteins in the cotyledons. By two days, the ADH activity in the cotyledons had dropped to almost negligible levels and the amounts of ADH activity in the roots showed a steady decrease up to four days postimbibition.

The ADH activity in the seeds and seedlings was within a similar range as that observed in seedling roots treated with hypoxia (Chapter II). This indicates that natural anaerobiosis may be occurring in the germinating seeds. However, it appears to be a developmental response, rather than a stress-induced one, since hypoxia treatment failed to increase the amount of ADH activity (Figure 5-6). The results of Morohashi and Shimokoriyama (1972; 1975) show that there are high levels of ADH activity in the absence of a true anaerobic stress situation. Raymond and co-workers (1985) showed that fermentation contributed significantly to the energy metabolism of pea and maize seeds after 12 hours aerobic imbibition (phase II). In turnip (*B. napus*) and cabbage (*Brassica oleracea*) seeds, which contain fatty reserves, they did not observe a significant contribution from fermentation under aerobic conditions. A similar trend was observed in lettuce, sorghum, rice, flax and sunflower, that do not contain starchy reserves. Thus, the phenomenon of natural anaerobiosis during the early stages of germination is not universal.

A western blot of ADH polypeptides was performed to determine if there was a relationship between the amount of ADH polypeptide and the level of ADH enzyme

activity (Figure 5-3 and 5-4) since previous studies (Chapters II and III) indicated that there may not be. Results from the present study again failed to demonstrate a clear relationship. The western blot indicated that there was a smaller increase in the amount of ADH protein than would be expected from the ADH activity data (Figure 5-3). More significantly, there was far less ADH protein in two day roots than would be predicted from the activity data. A more detailed comparison was carried out by measuring ADH activity in the samples used for the western blot (Table 5-1). It appeared from these data that there was less activity in the one day seed than the dry seed, which does not agree with the amount of ADH protein on the western blot.

In Chapter III, different isozyme profiles were seen in germinated seeds and roots. In this study, I examined changes in the ADH isozyme profile during seedling development. Figure 5-5 is a nondenaturing polyacrylamide gel of ADH isozymes in dry seed, germinating seeds and young seedlings. As well, samples of air and nitrogen-treated barley seedling roots were included as a comparison. In the dry seed and the 24 hour germinated seed, there was a single isozyme band present. This band was also common to the cotyledons after 24 hours, the radicles and the young roots (two days old). There was a second isozyme band in the cotyledons, at a lower intensity than the first one. At this point the cotyledons were still enclosed in the seed coat, and had not turned green. The second band persisted at a relatively low level of intensity, for two days, after which it disappeared. In the radicles of the germinating seeds, there was one isozyme present after 24 hours. After three days postimbibition, both the isozymes have disappeared from the root and cotyledon samples.

Evidence of developmental control of ADH expression has also been observed in rice. During seed development, all three isozymes were expressed (Xie and Wu, 1989). The ADH $_{2/2}$ form was detected very early in seed formation (grain-filling stage), although it was detected at much lower levels than the other two. At the later stages of seed development (ripening stage and mature dry seed), ADH$_{2/2}$ and ADH $_{1/2}$ bands gradually disappeared, suggesting that the activities of these enzymes were selectively lost during

grain maturation. In mature dry seeds, $ADH_{1/1}$ was the predominant isozyme expressed. After germinating in air for three hours, the homodimer of $ADH_{1/1}$ was the still the most prevalent isozyme expressed, although there was another fainter band, anodic to the first. In rice embryos germinated for 48 hours in the air, then submitted to anoxia for at least six hours, a second anodic band appeared. The intensity of the two anodic bands increased as time of anoxic treatment increased (Ricard *et al.*, 1986).

In Figure 5-6, the effects of 24 hours suboxia treatment on ADH activity in the seed and young seedlings at various stages are shown. There is a fairly high level of enzyme activity in dry seed, compared to the other samples. After 24 hours of suboxia treatment, the ADH activity in the N_2-treated one day old seed appeared to be suppressed or induced to a much smaller degree, instead of showing a strong induction. A similar pattern is seen in the two day old roots. Since the ADH activity assays were normalized based upon the protein content of the samples, the changes in ADH induction may be a result of changes in the protein content of the tissues. Significant induction of ADH does not occur until the seedlings are four days old. This may also suggest that *de novo* synthesis of the enzyme is required.

In conclusion, further comparison between ADH activity and ADH protein quantity failed to demonstrate a clear relationship. The activity of ADH varied with developmental stage during early seedling growth. This was associated with changes in the isozyme profile. The isozyme band that was lost first is the same band that was induced in response to flooding in the seedling roots. This isozyme is common to the three systems studied. During early development, the seedlings are non-responsive to anoxia stress, and in fact, N_2 treatment appears to suppress the ADH activity. The isozyme changes at the time may point to a requirement for the induction of the slow migrating isozyme.

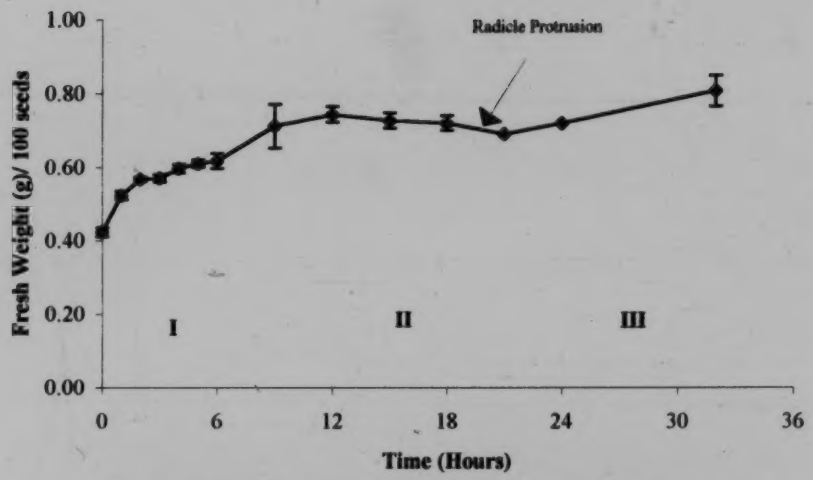


Figure 5-1. Time course of water uptake during seed germination.

Seeds were surface sterilized and germinated in petri plates of perlite in an environmental chamber. Each data point is the mean of three independent experiments (of two samples of 100 seeds each), with the standard error of the mean.

Figure 5-2. Seedling development from one to four days after imbibition.

The stages of seedling growth and development are illustrated; from one day (1-3 mm radicle protrusion) to four days after the begining of imbibition. After two days, the seed coat has been shed and the cotyledons are greening. Approximately three days after imbibition, the hypocotyl hook has straightened and the cotyledons are greened. By four days, the hypocotyl has elongated and the primary root has extended.

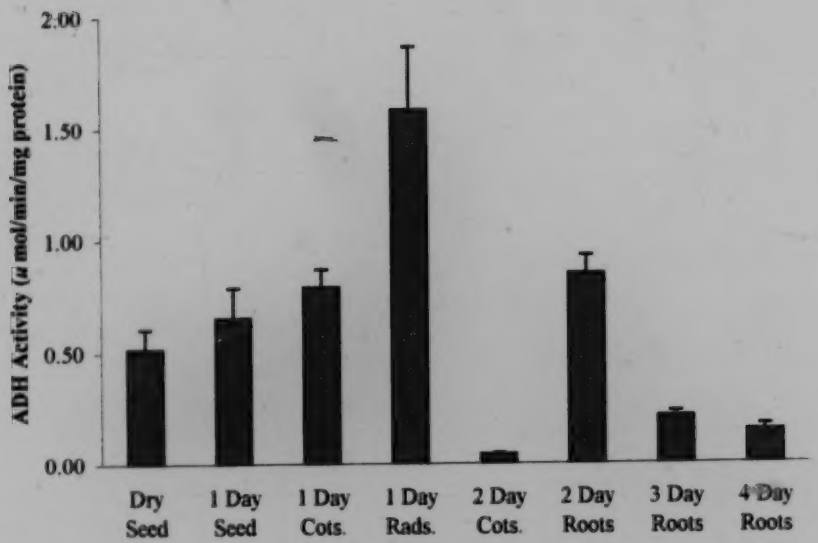


Figure 5-3. Alcohol dehydrogenase enzyme activity in germinating seeds and young seedlings.

Seeds were germinated in petri plates of perlite and grown for up to four days. The ADH enzyme activity was normalized on the basis of equal protein content of the samples. Each data point is the mean of three independent experiments of two samples each, with the standard error of the mean. (Cots.= Cotyledons, Rads.= Radicles)

Figure 5-4. Western Blot of ADH polypeptide in germinating seeds and young seedlings.

Seeds were germinated in petri dishes of sterilized perlite for up to four days. An equal amount of total protein (10 *u*g) was separated on a 12% SDS polyacrylamide gel (4% stacking gel) at 180 volts. The proteins were electroblotted onto supported nitrocellulose membranes at 75 volts for two hours at 4°C. The membranes were incubated in a 1:1000 dilution of primary ADH antibody. The size of the ADH subunit was determined by comparison with Bio-Rad Low Molecular Weight Standards (14.4 kD to 97 kD).

Lane #	Sample	ADH Activity (+/-SE) (umol/min/mg protein)
1	Dry seed	0.98 (0.09)
2	1 day seed	0.70 (0.13)
3	1 day radicles	1.69 (0.28)
4	2 day roots	0.77 (0.08)
5	3 day roots	0.19 (0.02)
6	4 day roots	0.16 (0.03)

Table 5-1. ADH activity in germinating seeds and young seedlings.

The ADH activity of the samples was normalized based on the protein content (Peterson, 1977) of the sample. Each data point is the mean of three determinations from each sample, with the standard error.

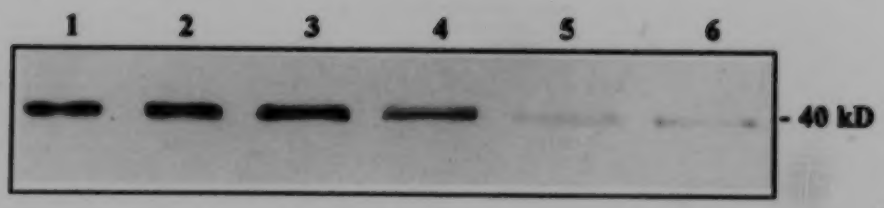
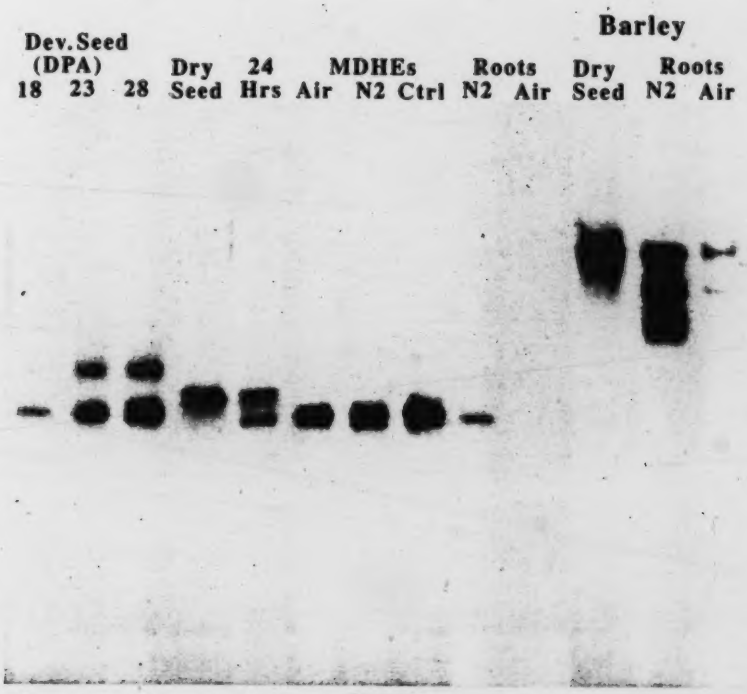

1
2
3
4
5
6
40 kD

Figure 5-5. Nondenaturing PAGE of ADH isozymes in germinating seeds and young seedlings.

Seeds were germinated in petri plates of sterilized perlite and grown for up to four days. Aliquots containing 50 *u*g of protein (barley seedling root samples: 25 ug) were separated on 1.5 mm, nondenaturing PAGE (7.5% acrylamide). The gels were run at 75 volts, at 4°C, in Laemmli running buffer (Laemmli, 1970) and stained (25°C) in a solution containing 7.6% ethanol, 452.2 *u*M NAD, 244.6 *u*M NBT and 163.2 *u*M PMS in 0.1 M Tris-HCl (pH 8.0). Samples of seedling roots of *H. vulgare* 'Leduc' were included for comparison. (Cots- cotyledons, Rads- radicles)


Barley
Dev. Seed
(DPA)
18 23 28
Dry Seed
24 Hrs Air
MDHEs N2 Ctrl
Roots N2
Roots Air
Dry Seed
Roots N2
Roots Air

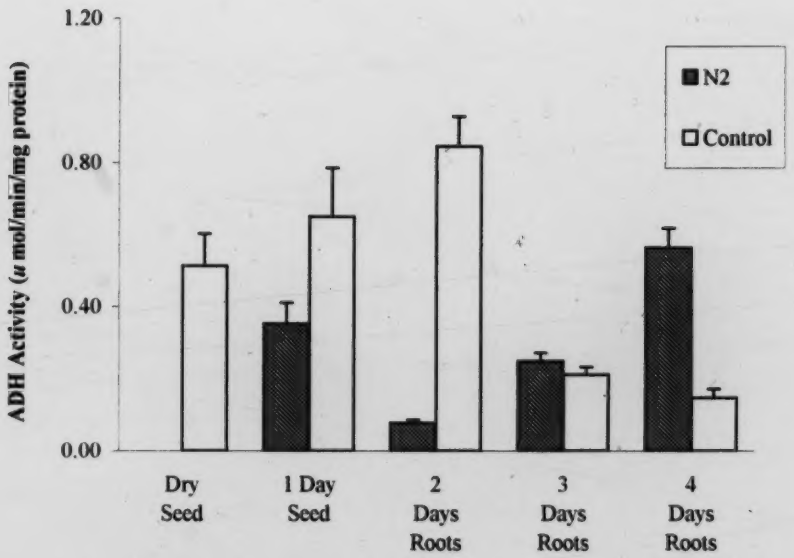


Figure 5-6. Alcohol dehydrogenase enzyme activity in germinating seeds and young seedlings after 24 hours suboxia treatment.

Seeds were germinated in petri plates of perlite in an environmental chamber. The seeds and young seedlings were treated for 24 hours. Control samples were grown on perlite in petri plates. Each data point was the mean of three independent experiments of two samples each, with the standard error of the mean.

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Chapter VI. Summary

Discussion

The studies reported herein focused on the characterization of the suboxic response in *Brassica napus*. This plant material is extremely important to the agriculture and agri-food industry in western Canada, and has received very little attention in terms of flooding tolerance. In addition, the microspore-derived haploid embryo (MDHE) system in *B. napus* is well established and warranted consideration as a system for selection of flooding tolerant genotypes. Four different tissue types; germinating seeds, very young seedling roots (< four days old) and older seedling roots (> four days old), and the *in vitro* MDHE system were examined to give a clearer picture of how *B. napus* would respond to suboxic conditions, and how developmental changes might impact upon that response.

In *B. napus* seedling roots, the response to hypoxia was one of acclimation or adjustment, leading to the maintenance of the AEC ratios and the TAs. These changes were both developmental and biochemical. The seedlings adusted to the hypoxic conditions by a reduced rate of growth and through the development of adventitious roots which showed evidence of the formation of aerenchyma tissue. The reduced growth rate may have contributed to the maintenance of the AEC ratio, while the morphological changes in the roots may have increased the diffusion of air to the root meristem. The effectiveness of the lacunae was apparent by the fact that root growth, while reduced, was not affected to the same extent as shoot growth. This was unusual, as most reports indicate a decrease in root growth in response to hypoxia (Wignarajah *et al.*, 1976; Thomson *et al.*, 1989; Huang *et al.*, 1994).

As expected, ADH activity was induced in the seedling roots under hypoxia. The ADH activity followed a biphasic pattern over the seven day treatment period. Increases in ADH activity with time have been reported in the literature for many different species (Wignarajah *et al.*, 1976; Jenkin and ap Rees, 1983; Cobb and Kennedy, 1987; Xie and

Wu, 1989; Good and Crosby, 1989; Andrews *et al.*, 1993, VanToai *et al.*, 1995). The biphasic pattern of ADH activity observed in the present study appeared to be temporally regulated as a similar pattern is seen in the control seedlings as in the treated ones. The temporal regulation of ADH activity is supported by the work of Lai and co-workers (1992), who show a biphasic induction pattern of ADH activity in a nondifferentiating tissue culture system.

Changes in ADH activity were not reflected in the quantity of ADH polypeptide as indicated by both western blotting and the nondenaturing gels. A possible model can be used to explain this phenomenon: if there was a pool of inactivated or sequestered enzyme, it could be activated as a 'quick or short term' response to the hypoxia treatment (up to about three days). If the ADH enzyme was stored in the monomer form, then the assembly and activation of the dimeric enzymes would not be detected as a change in ADH quantity on a western blot. This would also be true if the inactive enzyme was sequestered as a holoenzyme. The drop in ADH activity may indicate that the pool of available ADH was exhausted and had to be replenished through *de novo* synthesis.

The MDHE system was examined as a possible means of selecting for anoxia tolerance, and to gain further insight into the anaerobic response in *B. napus*. Unlike other *in vitro* systems (Vanlerberghe *et al.*, 1990; Lai *et al.*, 1992), ADH activity was quite high and was not further induced by anoxia. In both *in vitro* systems, anaerobiosis treatment resulted in a significant induction of ADH activity and/or ethanol accumulation (Vanlerberghe *et al.*, 1990; Lai *et al.*, 1992). The result in the MDHE system also contrasts with the response seen in seedling roots (Chapter II), where ADH activity increased sharply with hypoxia treatment. Interestingly, the level of ADH activity observed in the MDHEs was not significantly different to the maximum level of ADH activity in the seedling roots under hypoxia.

In contrast to previous work on the seedling roots (Chapter II) and other studies (Johnson *et al.*, 1989; Hole *et al.*, 1992; Johnson *et al.*, 1994), high levels of ADH

activity in the MDHE system did not result in maintenance of the TA levels and did not confer survival under anoxia. After nine days treatment, ADH activity is still relatively high, although survival of the MDHEs is very low. The size of the TA pool is decreasing and possibly the AEC ratio would show a decrease at day nine and beyond, although the AEC does not drop dramatically at day seven. The lack of change in the AEC ratio may be a result of adenylates being withdrawn from the pool. Regardless of the dynamics of the adenylates pool, these results clearly demonstrate that the high ADH activity was not sufficient to maintain the TA pool, nor did it confer survival to the MDHEs.

Further evidence supporting the lack of correlation between the ADH activity and the amount of ADH protein present was obtained from the MDHEs. This was also noted in the germinating seeds and young seedlings. While this analysis was not exhaustive, it does have implications with regard to experimental design of further research.

It appears that, developmentally, the MDHEs are similar to both seeds and seedling roots. When the ADH activity was compared to that in germinating seeds, the amount was similar to that seen in one day seed, one day old radicles and two day old roots, except that in seeds, N_2 treatment appeared to decrease or inhibit the induction of ADH activity. The isozyme data also point to similarities between the MDHEs, seeds and seedling roots, as they all share a common band.

Substantial variation in anoxia tolerance was observed in the MDHE population and on the basis of their response to anoxia, a selection was made *in vitro*. Unfortunately, this trait did not turn out to be stable and heritable past the second generation. There is evidence in the literature that plant tissue culture may result in somaclonal or gametoclonal variation, which appears in early generations, but is not stable and heritable (Larkin and Scowcroft, 1981). This may have occurred in the present study.

Results from both the seedling roots and the MDHEs suggest that the ADH activity may be both developmentally and temporally regulated. It could be argued that

the decrease in activity at three days in the seedling roots was a temporal response, while the lack of change in activity with time in the MDHEs, which were at the same developmental stage throughout the analysis, indicate that this was developmental regulation. In the seedling root system, the isozyme band was inducible under hypoxia, but induction was not seen in the MDHEs in this band. This was also the same band that disappeared from the profile of the germinating seeds (by two days in the cotyledons and three days in the roots), so it appears to be developmentally regulated in one system (the germinating seeds and early seedlings) and stress-induced in seedling roots at later stages of growth.

Seeds and young seedlings (less than four days old) were studied to gain further insight into this phenomenon of dual control of the anaerobic response. Again, it was observed that the induction of ADH activity changed with development. In other studies examining the developmental regulation of ADH activity, the authors have concluded that ADH increased in response to 'natural anaerobiosis' (Leblova, 1978; Raymond *et al.*, 1985; Botha *et al.*, 1992; Bewley and Black, 1994). The results presented herein show no evidence of this, while the changes appear to be developmental in nature.

There was a relatively high (compared to that in the MDHEs and seedling roots) level of ADH activity in the dry seeds, one day old radicles and cotyledons. At this point, the cotyledons were still enclosed by the seed coat, but the radicle had emerged and the high ADH activity may have been caused by an impedance of O_2 movement into the seed by the seed coat. Although it is possible that the cotyledons are experiencing some degree of hypoxia at this point, this is not likely a response induced by natural hypoxia since ADH is not induced by an imposed anoxia treatment at this stage. Further, any response measured thereafter was strictly developmental as germination was complete after 18 to 24 hours. Developmental changes were also observed in the inducibility of ADH activity. It was not inducible in the young seedling roots until three days postimbibition, and then only slightly. Intriguingly, the N_2-treatment in the two-day old

roots appeared to be inhibitory to ADH induction. After day four the ADH activity in the seedling roots was induced by hypoxia treatment.

From the isozyme gel data, there was a total of at least four unique bands present in the tissues examined. Allelic differences aside, this information, (which is by no means exhaustive), may indicate a possible model of a two or three gene *Adh* system. The slow-migrating band, common to the seeds, roots and very young cotyledons, is probably $ADH_{1/1}$ and the unique one in the germinating seeds (fastest migrating one) could be $ADH_{2/2}$, with $ADH_{1/2}$ observed only in the MDHEs. In maize seedlings subjected to anaerobiosis, there are three electrophoretically separable genic isozymes of ADH induced in the roots (Freeling and Schwartz, 1973). These three isozymes are formed by the random dimerization of the polypeptides encoded by two different *Adh* genes, *Adh*1 and *Adh*2. Set 1 enzymes ($ADH_{1/1}$) migrate most slowly towards the anode; Set III ($ADH_{2/2}$) isozymes migrate the fastest, and Set II ($ADH_{1/2}$) isozymes move at an intermediate rate (Freeling and Bennett, 1985).

Conclusions

It appeared from this work and that of others that there was a strong relationship between ADH activity and the AEC ratio and TA pool size under hypoxia in the seedling roots, but the relationship is not as clear cut in the MDHEs under anoxia. It also appeared that *de novo* synthesis may not be required for induction of ADH in the seedling roots, at least for a short term response, but may be required for induction in a longer period of hypoxia treatment. Whether or not ADH is required for survival is still debatable. In the MDHEs, it does not seem to be a factor in survival (or lack thereof). Possibly there are other factors at work in this system which are responsible for the lack of survival, such as the occurrence of acidosis, a build up of toxic metabolic byproducts, or perhaps the TA pool is depleted. The seeds do not appear to be experiencing a 'natural anaerobiosis', as

observed in other work. There was more evidence pointing towards a developmental switch which occurred between day two and three postimbibition.

The results from this study demonstrate that regulation of the suboxia response are more complex than first thought. The implications of this are very important when considering approaches for selecting for flooding tolerance. In many growing areas, the threat of flooding occurs during seed germination and early seedling development. It is apparent that *B. napus*, and possibly other crop plants do not rely solely on ADH activity to survive the stress.

Future Directions

Future work would help to elucidate the relationships between ADH activity, the AEC ratio and TA pool size and survival under anoxia, as well as the regulation of ADH induction. There is also a need for a more intensive study of the genetics of the *Adh* genes in *Brassica napus*, and the developmental regulation and morphological changes that occur under suboxic conditions. In the seedling system, a study of the pattern of *de novo* proteins synthesized over the treatment period might answer some important questions about the response to hypoxia. As well, an examination of ethylene biosynthesis during adventitious root and aerenchyma tissue development might yield information concerning the role of ethylene during induction. In the germinating seeds, a study of the isozyme pattern under anoxia, as well as the AEC ratio and TA during germination and early seedling growth would also be useful. In addition, it would be interesting to look for *Adh* mRNA induction in the germinating seeds and young seedlings to further characterize the regulation of the suboxia response.

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Appendix

ANOVA Table: ADH Activity in Seedling Roots

Treatments	Count	Sum	Average	Variance
Day 0	3	3.24	1.08	0
1	3	16.38	5.46	2.80
2	3	16.42	5.47	24.12
3	3	8.56	2.85	7.07
4	3	15.47	5.16	15.73
5	3	13.17	4.39	16.72
6	3	13.82	4.61	20.33
7	3	10.16	3.39	8.36
Control	8	27.46	3.43	2.63
Air	8	10.82	1.35	0.86
N_2	8	58.94	7.37	9.37

Source of Variation	SS	df	MS	F	P-value	F critical
Between Days	49.07	7	7.01	2.40	0.078	2.76
Between Treatments	149.31	2	74.65	25.53	0.000	3.74
Error	40.93	14	2.92			
Total	239.31	23				

ANOVA Table: AEC in Seedling Roots

Treatments	Count	Sum	Average	Variance
Control	5	4.37	0.874	0.002
Air	5	4.26	0.852	0.001
N_2	5	4.30	0.860	0.001

Source of Variation	SS	df	MS	F	P-value	F critical
Between Treatments	0.0012	2	0.00062	0.477	0.632	3.885
Within Treatments	0.0156	12	0.0013			
Total	0.0168	14				

ANOVA Table: ADH Activity in Haploid Embryos

Treatments	Count	Sum	Average	Variance
N_2	9	7.51	0.84	0.013
Control	9	8.46	0.94	0.065

Source of Variation	SS	df	MS	F	P-value	F critical
Between Treatments	0.050	1	0.05	1.27	0.276	4.494
Within Treatments	0.62	16	0.039			
Total	0.0673	17				

ANOVA Table: AEC in Haploid Embryos

Treatments	Count	Sum	Average	Variance
Control	4	2.93	0.73	0.005
Air	4	2.93	0.73	0.006
N_2	4	2.85	0.71	0.006

Source of Variation	SS	df	MS	F	P-value	F critical
Between Treatments	0.001	2	0.001	2.40	0.088	4.256
Within Treatments	0.055	9	0.006	25.53		
Total	0.056	11				